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Phytochemical Investigation and Drug Interaction Potential of *Cyanotis vaga*

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PHYTOCHEMICAL INVESTIGATION AND DRUG INTERACTION POTENTIAL OF *CYANOTIS VAGA*

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ABSTRACT

Cyanotis vaga (Lour.) Schult, & Schult. f. (*C. vaga*) previously called *Tradescantia vaga* and *Cyanotis barbata*, is an annual to perennial herb, belonging to the family Commelinaceae. It is native to Eastern Asia and Tropical Africa. Also, it is common in all types of forests, especially in pine forests, as well as in grassy areas. It has been reported that its decoction reduces fever and enhances well-being. Recently, *C. vaga* has been used widely for its anabolic effects, due to its richness of ecdysteroid compounds. Remarkably, all isolated compounds from *C. vaga* in this study are ecdysteroids. Even though *C. vaga* is a common medicinal plant, there are no reports about its effect on the drug metabolizing enzymes (cytochromes P450) to predict its interaction with concomitantly used medications.

In this study, eight ecdysteroids were isolated from *C. vaga*, including two previously reported from *C. vaga*: 20-hydroxyecdysone (**1**) and rubrosterone (**2**). The additional six ecdysteroids were elucidated for the first time from *C. vaga*, and identified as 20-hydroxyecdysone 2-acetate (**3**), 20-hydroxyecdysone 3-acetate (**4**), 2-doxyrubrosterone (**5**), poststerone (**6**), ajugasterone C (**7**) and dacryhainansterone (**8**). The plant extract and isolates were tested for their inhibitory effects on the CYP3A4 enzyme, which is a major CYP isoform involved in the metabolism of commonly-used clinical drugs. The extract showed strong inhibition of CYP3A4 with an IC_{50} value of 8.5 μg/ml, while compounds **2**, **3**, and **8** showed moderate inhibition for CYP3A4.

DEDICATION

To my parents, my wife, my daughters, my sisters, my brothers, and to everyone who loves to hear that I have achieved something.

ACKNOWLEDGMENTS

I would like to thank every single person who contributed by advising, revising or any form in this work to make me able to complete my thesis. I want to thank and recognize my supervisor Dr. Ikhlas A. Khan for giving me an opportunity to join his research laboratories and for his help and support in this most challenging time that made me build and develop my academic skills. I am thankful for my committee members – Dr. Samir A. Ross, Dr. Robert J. Doerksen, and Dr. Sudeshna Roy – for their time and for serving in my committee. My thanks extend to Dr. Zulfiqar Ali for his guidance in my research work and his open door all the time, as well as Dr. Shabana Khan for conducting cytochrome P 450 inhibition assay in her laboratory, and Dr. Bharathi Avula for running HR-MS for my samples. I am thankful to the project coordinator, Jennifer Taylor, for her help all the time. Thank you to my professors in the BioMolecular Sciences Department, staff members, and graduate students. Special thanks to Dr. Khan's graduate students, Omer Fantoukh and Mohammed Hawwal and other Saudi students, for helping me in my first-year seminar, advising, and standing with me all this time. Thank you to the Graduate Writing Center, as well as Dr. Jon F. Parcher and Dr. Mohammed Albadry for helping me edit my thesis to appear in its revised state. I would like to thank my laboratory mates whom I had a chance to learn from Dr. Ahmed Galal, Dr. Ahmed Awad, Dr. Mustafa Ghanadian, Ebru Erol, Taghreed Majrashi, Abidah Parveen, Pei Cee, and Abdulfatai Ajiboye. Again, thank you to every single person who contributed by advising, revising, or in any kind of supports.

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INTRODUCTION

1.1 Natural Products Background

The significance of natural products on human health issues is undeniable. Plant-based natural products represent 25% of all prescribed pharmaceuticals in industrialized countries. Moreover, flowering plants exclusively deliver 11% of the WHO's list for primary and essential medications (Namdeo, 2007). Plant-based natural products, which are traditionally considered as secondary metabolites, are compounds that primarily do not contribute directly in either growth or development of the plant, and they may not have a recognized role in fundamental life processes. They are distributed and categorized among the plant kingdom in a way that shows distinct botanical taxonomy groups. However, primary metabolites (for example nucleotides, organic and amino acids) are present in all plants and play essential metabolic roles. Biosynthetic origins and chemical structures of primary and secondary metabolites share many similarities which make use them unhelpful to distinguish between metabolite categories. On the other hand, functionality can be a valid distinction between primary metabolites, which contribute to nutrition, growth, and development, and secondary metabolites that are influenced by environmental interaction with plants (Croteau, Kutchan, & Lewis, 2000; Namdeo, 2007). In addition to numerous drugs, drug candidates and biological activities that secondary metabolites provide, plant-based natural products (secondary metabolites) are the most diverse category of dietary supplements. It is also one of the most un-prescribed alternative medicine, along with vitamins (De Smet et al., 2000).

According to the Dietary Supplement Health and Education Act of 1994 (DSHEA), dietary supplements must state that the product is not intended to diagnose, treat, cure, or prevent any disease. Those include vitamins, minerals, amino acids, herbs or other botanicals, supplements that are used to increase total dietary intake, metabolites, extracts, or combinations of any ingredient described here (Health government, 1997; Newman & Cragg, 2007). The Act (DSHEA) enhanced the growth of herbal products as dietary supplements and limited the control of the Food and Drug Administration over them. As a result of this, the use of herbal remedies increased in the United States by 380% between 1990 and 1997 (Morris & Avorn, 2003). Obtaining herbal products from the internet is common, as it does not require healthcare provider approval (Owens, Baergen, & Puckett, 2014).

In this study, an example of an online herbal dietary supplement, *Cyanotis vaga,* will be investigated phytochemically and will be tested for the possibility of interacting with concomitantly used medications. Through a quick online search on *Cyanotis vaga*, there are many products and different dosage forms available for this plant. The main users of *C. vaga* products are athletes, due to its ecdysteroids compounds. In this manuscript, *C. vaga* will be studied from different aspects in two chapters. The first chapter will cover *C. vaga* and ecdysteroids, in addition to the thesis objectives. The second chapter will take an in-depth approach to discuss *C. vaga* phytochemicals; then the plant extract and the isolates will be tested biologically.

1.2 Overview of *Cyanotis vaga*

Cyanotis. vaga (Lour.) Schult & Schult.f., commonly known in India as Wondering Dew-Grass, belongs to the Commelinaceae family, which has about 700 species under 50 genera broadly distributed in tropical and subtropical regions. *C. vaga* was called *Tradescantia vaga* from 1790 to 1870 "TYPE: Cantona, China" then *Cyanotis barbata* from 1825 to 1894 "TYPE: Nepal" (Flowers of India, 2013; Nandikar & Gurav, 2014). According to The Plant List database, there are 16 different names, not including *C. barbata*, that are considered synonyms (The Plant List, 2013); however, currently the only accepted name is *Cyanotis vaga*.

1.2.1 Description

Cyanotis vaga is an annual to perennial plant, rooting at the lower nodes allowing a new plant to rise with stems fully branched typically from the base, or distally, or few branched, 10-60 cm. Leaves are on lateral branches with oval to lanceolate margins (10–15 × 0.5 cm, apex acute, base rounded, margin entire, serceous, leaf sheath 0.3–1 cm long). Flowers are bisexual, sepals oblanceolate to oblong (0.4–0.6 × 0.2 cm, pilose or glabrous; petals blue-purple or violet (**Figure 1-1**) and (**Figure 1-2**) (Nandikar & Gurav, 2014; World Flora Online, 2019).

Figure 1-1 *C. vaga* (Lour.) Schult & Schult.f.(Nandikar & Gurav, 2014)

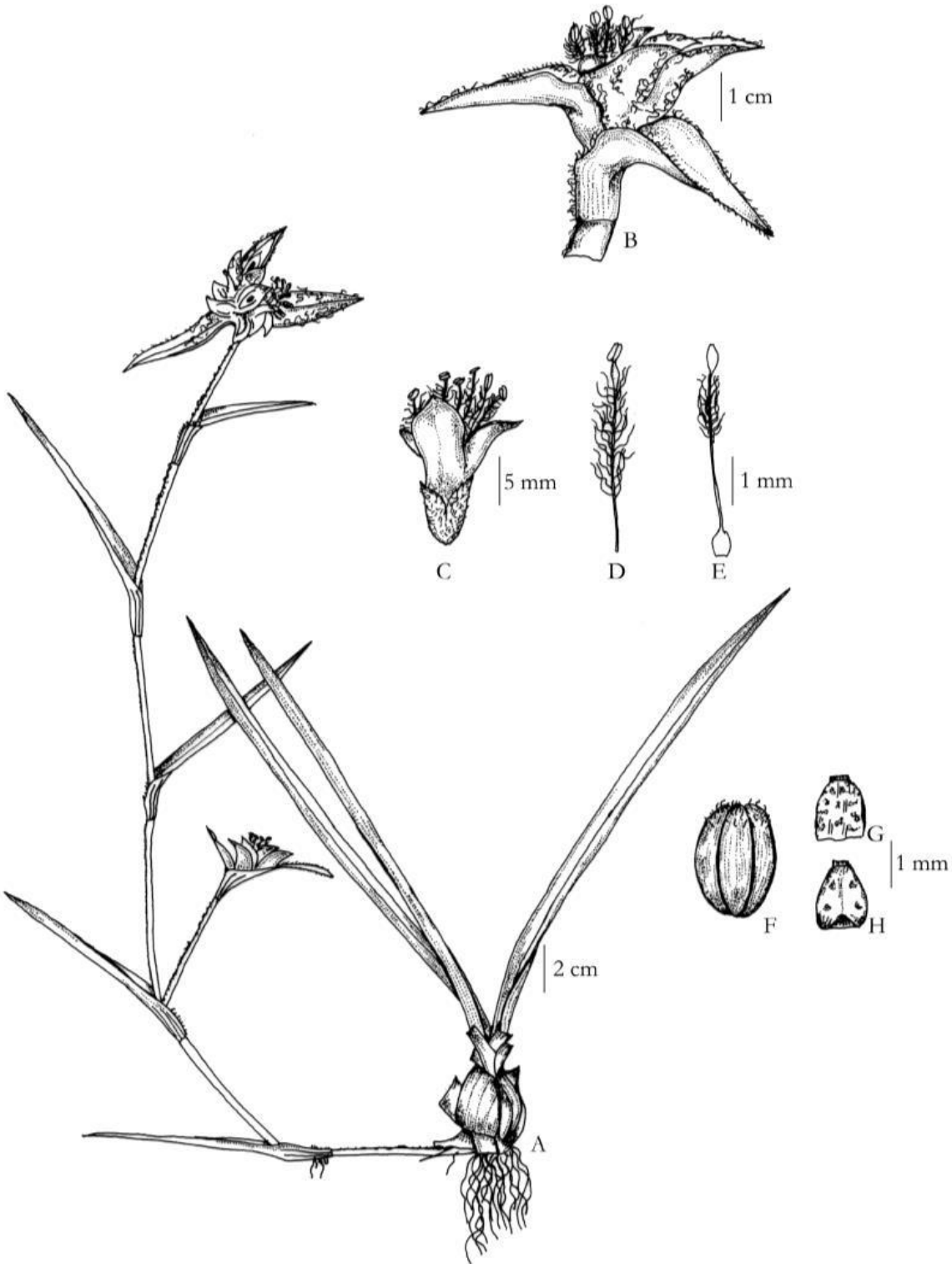


Figure 1-2 *Cyanotis vaga* (Lour.) Schult. and Schult. f. A: Habit, B: Inflorescence, C: Flower, D: Stamen, E: Pistil, F: Capsule, G–H: Dorsal and ventral view of seed. Drawn by Mayur Nandikar from M.D. Nandikar 1230 (SUK).

1.2.2 Distribution

C. vaga has been a common species since the 19th century in Eastern Asia, which was described by Don (1825) as *Cyanotis barbata* D. Don. It is native to northeastern parts of India, Nepal, and Taiwan, in addition to Thailand and Vietnam. *C. vaga* is common in different types of forests, mainly in pine forests as well as in grassy areas. The flora of China also mentions *C. vaga* as a native Chinese plant (Tropicos.org., 2019). Furthermore, *C. vaga's* scope of distribution extends to reach Tropical Africa and the Southwest of the Arabian Peninsula(Nandikar & Gurav, 2014; Tropicos.org., 2019) (**Figure 1-3**).

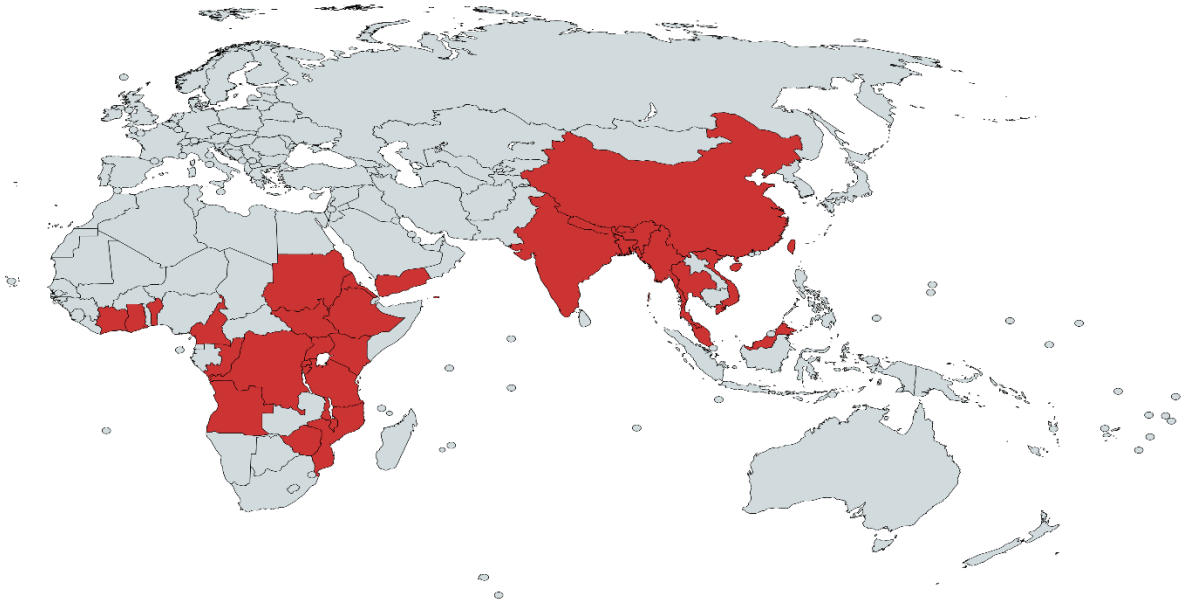


Figure 1-3 *C.vaga* distribution, Red color indicates native regions; eastern Asia, Tropical Africa, and SW. Arabian Peninsula

1.2.3 Medicinal uses and reported Biological activities

It has been reported that *C. vaga's* decoction reduces fever and enhances well-being(Santos, Chua, Eufemio, & Abela, 1970). Also, its crude extract has shown antimutagenic effects(Gagalac-Nicolas & Lim-Sylianco, 1981). Several references, in 2006, 2015, and 2016 showed extensive studies on *C. vaga* used in various formulae in Chinese medicines. *C. vaga*, mainly roots, has been used in those formulae along with other plants to treat several health conditions. Those conditions could be specific such as freckle removing(Tong & Li, 2016), postpartum perineal laceration, and tear scars prevention(C. Li, 2016; Su, 2016), treating femur head necrosis(Zhuang & Wang, 2016), viral myocarditis treatment(Bian, 2015), eyelashes edge inflammation treatment(Zhang, 2015), or a granule for treating anemia(Zhou, 2015). Other preparations indicate for dampness elimination and pain relieving(Lan, 2006), treatment of vasospasm in a spray form(Pang, 2015), and disinfectant for operating room(Wang, 2016) as well as a drug formulated for otitis media (D. Li, 2016). It has been published in patents for all of the previously listed conditions, and the amount of *C. vaga* and other plants to prepare the formulae differ from one another. Recently, *C. vaga* has been widely used for its anabolic effects due to the richness of its ecdysteroid compounds which have anabolic growth-stimulating activity on different *in-vivo* models such as mice, Japanese quails, and pigs. (Borrione, Luigi, Maffulli, & Pigozzi, 2008). Remarkably, all isolated compounds from *C. vaga* in this study are ecdysteroids. This class of secondary metabolites will be discussed in detail from several aspects in the next section.

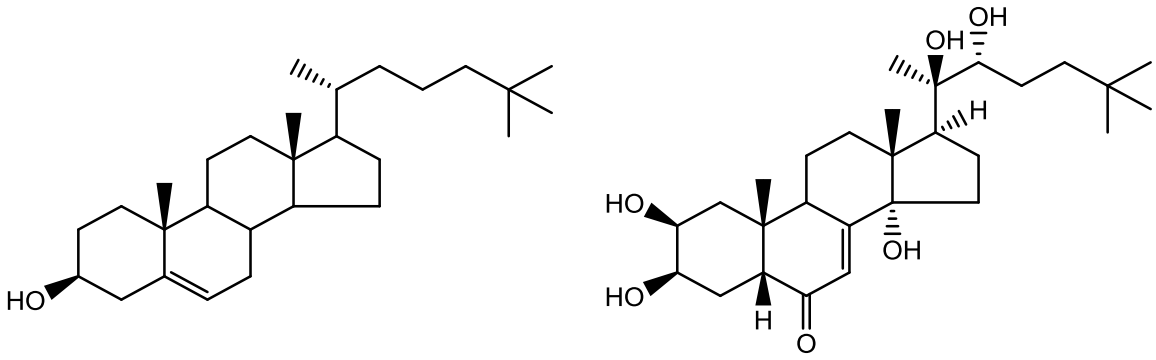
1.3 Ecdysteroids Class Overview

Ecdysteroids are secondary metabolites, found in different living organisms, known as insect molting hormones. Phytoecdysteroids refers to ecdysteroids obtained from plants, whereas Zooecdysteroids are isolated ecdysteroids from arthropods(Hetru & Horn, 1980). The discovery of ecdysteroids 65 years ago, led companies to invest millions of dollars in the 1980s, hoping to find a proper chemical pest control that came from a natural source, had a new biological target site and was safe for the environment. Ecdysteroids, arthropods' steroid hormones, regulate molting, metamorphosis, and reproductive functions. They are, however, not anticipated to bind to the mammalian steroid receptors due to their slight chemical structure differences (Laurence Dinan, 1995; Lafont & Dinan, 2003). The presence of ecdysteroids in plants in large amounts, compared to very low yield in insects, increases the number of biological studies to investigate their effects and benefits on humans as well as animals(Chandrakala, Maribashetty, & Jyothi, 1998).

1.3.1 Chemical structure and biosynthesis

Ecdysteroids share some common chemical components. The backbone of ecdysteroids typically consists of 27, 28, or 29 carbons, and cannot be fewer than 19 carbons. Normally, they have a 7-en-6-one chromophore in ring B and a hydroxyl group at position 14. Also, the other characteristic feature is an A/B-cis ring fusion. Carbon 17 side chain usually controls the number of carbons in the structure. Hydroxylation of positions 1, 2, 3, 5, and 11 is common in addition to the main hydroxyl group at position 14. The free state of ecdysteroids is the dominant case; however, glycosides, esters, and ethers derivatives have been found(Baltaev, 2000; L. Dinan, 2001).

The major biosynthetic pathways of plants' ecdysteroids (phytoecdysteroids) have been proven. 20-hydroxyecdysone, the most abundant ecdysteroid compound in both insects and plants, is the main compound in the biosynthesis studies. These studies experimentally confirmed that the cholesterol is the precursor for phytoecdysteroid. The7-en-6-one chromophore arises along with the A/B-Cis ring fusion in the beginning steps of phytoecdysteroids biosynthesis. The next process is a hydroxylation at 14α position. The determination of other hydroxylations' introduction order into the ecdysteroids backbone is not rigorously regulated as it can be different from plant to another(Baltaev, 2000; Davies, Lockley, Boid, Rees, & Goodwin, 1980)(**Figure 1-4**).



Cholesterol

20-hydroxyecdysone

Figure 1-4 cholesterol and 20-hydroxyecdysone structures

1.3.2 Ecdysteroids in plants

The first ecdysteroid from plants was isolated in 1966, 12 years after the isolation of α-ecdysone from pupae of silkworm(Butenandt & Karlson, 1954; Nakanishi, Koreeda, Sasaki, Chang, & Hsu, 1966). More than 500 ecdysteroid analogs have been reported between 1966 and 2017(Lafont R., 2017). All plant parts – roots, stems, leaves, flowers, and seeds – contain phytoecdysteroids; however, the amount of them vary depending on the plant growing stage(L. Dinan, Savchenko, & Whiting, 2001). Although phytoecdysteroids are arthropods reproduction

hormones, they share many features with plant growth hormones, brassinosteroids. By contrast, phytoecdysteroids, in general, do not induce responses from the classical plant hormones(Gorelick-Feldman et al., 2008). The use of phytoecdysteroids on the hormonal level, mainly the growth and reproductions, is not totally approved. Nevertheless, a study on the level of 20-hydroxyecdysone among different plants organs reveals that reproductive organs possess the highest level of 20-hydroxyecdysone, and the aerial parts accumulate the maximum amount of phytoecdysteroids during an intense growth period(Zibareva, 2000).

There is a robust amount of literature reporting the defense role of phytoecdysteroids in plants since they can defeat insect attacks on plants by acting as antifeedant substances, deterrent effects or endocrine-disrupting toxins(Bajguz & Dinan, 2004; L. Dinan, 2001; Soriano, Riley, Potter, & Bowers, 2004). Increasing the concentration of phytoecdysteroids is a proven strategy to rid an area of arthropods. Schmelz (1999) concluded that phytoecdysteroids concentration increased three fold in the root of *Spinacia oleracea* when it was exposed to root herbivory by Black Vine Weevil (BVW) larvae(Schmelz, Grebenok, Galbraith, & Bowers, 1999). Soriano (2004) showed the effect of elevated 20-hydroxyecdysone in *Spinacia oleracea* on four nematode species. Phytoecdysteroids, specifically 20-hydroxyecdysone, has protected the plant and led to molting abnormality, reduced invasion, and death of nematodes(Soriano et al., 2004).

1.3.3 Therapeutic properties

Ecdysteroids have been investigated for biological activities since they were discovered(Burdette & Richards, 1961). The effects of ecdysteroids on mammalians are diverse and affect many bodily functions constructively(Bathori, Toth, Hunyadi, Marki, & Zador, 2008). The biological activities of ecdysteroids have been reviewed in numerous articles, which are

mentioned most extensively in 2003 by Lafont R. and Dinan L(Lafont & Dinan, 2003). The most recent and brief review of ecdysteroids applications was done by Al Nagger et al. (2017) who summarized the pharmacological effects of ecdysteroids on physiological functions. Ecdysteroids have shown anabolic and adaptogenic activities, as well as hypoglycemic and hypocholesterolemic effects. Additional wound-healing and immuneprotectant abilities were demonstrated on mice, rats, and humans(Al Naggar, Ghorab, & Mohamed, 2017). Parr et al. (2015) carried out a study to investigate a hypothesis of estrogen receptor (ER) for ecdysteroids using in-silico modelling and to evaluate the anabolic *in-vitro* potency of ecdysteroids compared to other recognized anabolic chemicals and gave a suggestion about the consideration of ecdysteroids as prohibited substances of the World Anti-Doping Agency (WADA). She found that in-silico docking data of ecdysteroids' anabolic activity is mediated through the estrogen receptor β- subtype. Also, her data showed that ecdysteroids' anabolic potency were equal or higher than the anabolic androgenic steroids. Based on the high ecdysteroids anabolic potency in the rats, the author justified her suggestion to list ecdysteroids in the prohibited substances in the "S1 Anabolic Agents" category(Parr et al., 2015). According to the website of the World Anti-Doping Agency, a project was funded in 2016 to study the effects of ecdysteroids-containing products on human athletic performance(World Anti-Doping Agency, 2016)

1.4 THESIS OBJECTIVES

Cyanotis vaga is a tremendous source of ecdysteroids. The anabolic effects of ecdysteroids increase the marketing of plants containing ecdysteroids as dietary supplements. The main focus of this study on ecdysteroid activities is due to their anabolic effect, which is the target of *C. vaga* consumers. Ecdysteroids do not bind to the androgen receptor (AR), which is the anabolic-androgenic steroids binding site — the main lead of increasing muscle mass. This binding difference makes ecdysteroids an excellent anabolic alternative as they lack anabolic-androgenic steroids' side effects, such as hepatotoxicity and behavioral changes(Bathori et al., 2008; Shahidi, 2001).

In this study, eight compounds isolated from *C. vaga* were ecdysteroids. This class of natural products, as discussed earlier, possesses anabolic activity which makes *C. vaga* an excellent candidate to be an herbal dietary supplement, as a rich source of ecdysteroids. Dietary supplements' advertisements demonstrate their potential benefits without revealing their side effects. Generally, herbs are assumed to be safe since they are natural. However, drugs and dietary supplements enter the body as foreign chemicals. They are metabolized mainly by cytochromes P450 enzymes. Vice versa, cytochromes P450 (CYP) enzymes are subjected to be induced or inhibited by drugs and dietary metabolites. There are 50 enzymes belonging to cytochrome P450. However, only six of them metabolize 90% of medications. CYP3A4 and CYP2D6 are the most significant isoforms(Lynch & Price, 2007).

Surprisingly, there are no reports about *C. vaga* effects on drug metabolizing enzymes, Cytochromes P450, to predict its interaction with concurrently administered medications. In this study, preliminary research of *C. vaga's* safety profile will be conducted(Borrione et al., 2008). First, phytochemical investigations of *C. vaga* will be initiated to be assured of its

ecdysteroids content and examine its constituents along with the plant extract against one of the most significant Cytochrome P450 metabolizing isoforms (CYP 3A4).

CHAPTER 2
Isolation, Elucidation, and Drug Interaction Assay of *C. vaga* and its Chemical Constituent

2.1 Introduction

The aims of this study, as mentioned earlier, were to investigate *C. vaga* phytochemically and to examine the plant extract and its constituents against one of the primary metabolizing enzymes (CYP 3A4). The chemical constituent's data available for *C. vaga* is very limited. First phytochemical study on *C. vaga* was done in 1970, and one ecdysteroid compound (Commisterone, currently called 20-hydroxyecdysone) was reported(Santos et al., 1970). Also, research groups in the Philippines worked on *C. vaga* in the 1970s and 1980s, and they reported two compounds. Another ecdysteroid compound (Rubrosterone), was isolated, in addition to a macrocyclic hydrocarbon(Abela & Santos, 1974; Chua, Santos, Abela, & Wagner, 1982). There are no reports about *C. vaga* and its constituents in terms of safety, or on drug-herbal interaction specifically. This project is the first study that was conducted on *C. vaga* and its chemical constituents against cytochrome P450 (3A4 isoform).

2.2 Experimental sections

2.2.1 General experimental procedures

NMR data were obtained on a Bruker 400 MHz and 500 MHz NMR spectrometers, whereas HR-ESI-MS spectra were recorded on an Agilent Series 1100 SL mass spectrometer. Chromatography was performed by silica gel (40 μm for flash chromatography, 60 Å, J. T. Baker),

Sephadex LH-20 (Sigma), and reversed-phase RP-C18 silica gel (Polarbond, JT Baker). TLC was checked under UV-254 nm, on aluminum-backed plates pre-coated with silica gel F254 (20 × 20 cm, 200 μm, 60 Å, Merck), then visualized by spraying with (5:95) vanillin solution in concentrated H_2SO_4-EtOH (Sigma), and dried by heat gun.

Recombinant human P450 3A4 (CYP3A4) was obtained from BD Gentest (Woburn, MA). Pooled human liver microsomes (HLM) prepared from 25 donors (total P450 concentration 0.320 nmol mg^{-1} and protein concentration 20 mg mL^{-1}) were bought from Corning Life Sciences (Cambridge, MA). 3-Cyano-7-ethox-ycoumarin (CEC), 7-methoxy-4-trifluoro-methylcoumarin (MEC), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), ketoconazole and 0.5 M potassium phosphate buffer (pH 7.4) were from BD Gentest (Woburn, MA). NADPH regeneration solutions A and B were obtained from Corning Discovery Labware (Woburn, MA). Acetaminophen, testosterone, reduced glutathione (GSH), salicylamide, 6b-hydroxy-testosterone, magnesium chloride, phenacetin, and corticosterone were from Sigma-Aldrich (St Louis, MO). Troleandomycin was from Santa Cruz Biotechnology, Inc. (Dallas, TX).

2.2.2 Plant material

Whole Plant material of *Cyanotis vaga* was bought from Badmonkey Botanicals in September 2014 (www.badmonkeybotanicals.com), authenticated and registered at the National Center for Natural Products Research (NCNPR), School of Pharmacy at the University of Mississippi (Voucher no 16840)

2.2.3 Isolation and fractionation

(10 g) of the whole plant (90% ethanol) extract was fractionated, and isolated by using different chromatographic stationary phases: silica gel 60 (230–400 mesh ASTM)**,** silica gel reverse phase C-18**,** Sephadex LH-20 and eluted with different solvent systems which then affords eight ecdysteroids, some of them reported for the first time from this species. In particular, the plant material (10 g) was subjected to silica gel column chromatography (CC) using elution gradients ($CHCl_3$-MeOH) starting with nonpolar toward polar gradients (10:0, 9.8:0.2, 9.5:0.5, 9:1, and 8.5:1.5) then (EtOAc-MeOH-H_2O), ratio (8:2:0.25), and finally 100% methanol, to give 34 fractions (F-1 to F-34). Fraction F-9 (286 mg) was further separated by CC over silica gel 60 (3cm x 118cm) eluted with (EtOAc-$CHCl_3$-MeOH-H_2O) (15:8:2:0.25) to obtain 6 sub-fractions F9A1 to F9A6. Compound **5** (2.2mg) was purified from sub-fraction F9-A2 by Reverse Phase C-18 (3cm x 25cm) eluted with stepwise gradient of water-methanol (7:3, 6:4, 1:9). F15 was subjected to CC over Sephadex LH-20 (5cm x 70cm) to collect F15B1 to F15B5. Sub-fractions F15B2 and F15B3 then subjected to CC over silica gel 60 to afford compounds **3** (9.4mg) and compound **8** (40mg), however, sub-fraction F15B5 afforded compound **6** (13mg) through reverse phase C-18 CC experiment. Compounds **2** and **4** were obtained from F18 by repeated CC over silica gel 60 (2.5cm x 110cm), eluted with (EtOAc-$CHCl_3$-MeOH-H_2O) (15:8:4:1), followed by reverse phase C-18 (34cm x 2cm) eluted with stepwise gradient of H_2O-MeOH (7.5:2.5, 6.5:3.5) to yield (10mg) of compound **2** and (8mg) of compound **4,** respectively. Finally, F23 was subjected to CC over silica gel 60(3cm x 118cm), eluted with (15:8:2:0.25) (EtOAc-$CHCl_3$-MeOH-H_2O) afforded compound **1** (579 mg) and compound **7** (211mg) as the two major compounds (**Figure 2-1**).

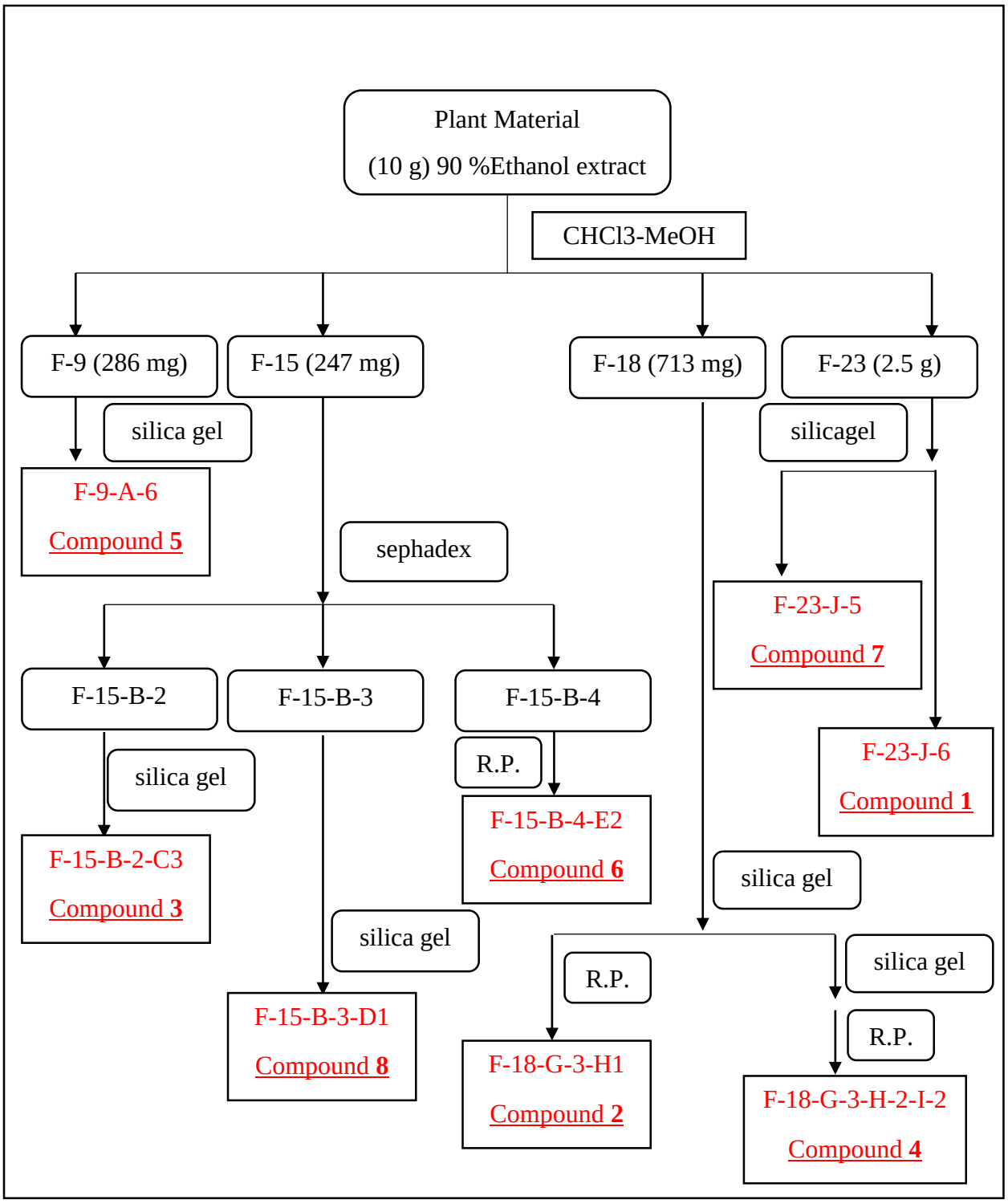


Figure 2-1 Isolation process for chemical constituents of C. vaga

2.2.4 Cytochrome P 3A4 Inhibition assay

The reversible inhibition of recombinant cytochrome P 3A4 was determined using Biological Fuel Cell (BFC) 10 mM, and assay conditions described earlier(Crespi, Miller, & Penman, 1997). Test sample and positive control were serially diluted in a solution (100 mL) of cofactors mix (1.3 mM NADP+, 66 mM MgCl2, and 66 mM G6P), control protein (15 mg ml^{-1}) and glucose-6-phosphate dehydrogenase (40 Units mL1 in 5 mM sodium citrate buffer) to achieve six concentrations in the range of 100–0.4 mM or mg ml^{-1}. After incubating at 37 °C for 10 min, the plates were read to record the inherent fluorescence of the test compounds (if any). The reaction was started by the adding enzyme substrate mixture (100 µL), followed by incubation for 30 minutes. The reaction was ended by adding 75 mL of ice-cold acetonitrile/0.5 M Tris base (80:20). Fluorescence was measured on Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA) at specified excitation and emission wavelength 409/530 nm for BFC/CYP3A4. IC_{50} values were generated by plotting test concentration versus % inhibition.

2.3 Results and discussion

2.3.1 Structures elucidation

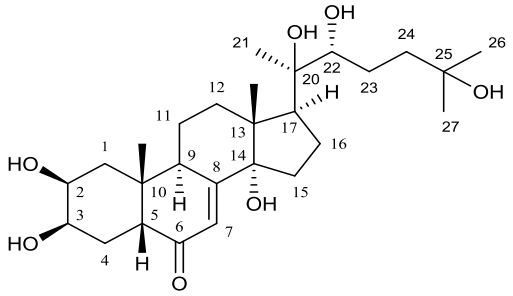
Structure elucidation of the isolated ecdysteroids (**Figure 2-2**) was accomplished by a combination of HR-ESI-MS and NMR data, in addition to the comparison of data from the literature. The compounds were identified as 20-hydroxyecdysone (**1**)(Budesinsky, Vokac, Harmatha, & Cvacka, 2008), Rubrosterone (**2**) (Tan, Wang, & Li, 2003) 20-hydroxyecdysone 2-acetate (**3**), 20-hydroxyecdysone 3-acetate (**4**) (Budesinsky et al., 2008), 2-deoxyrubrosterone (**5**) (Sydykov & Segal, 1976), Poststerone (**6**) (Tan et al., 2003) Ajugasterone C (**7**) (Budesinsky et al., 2008), and Dacryhainansterone (**8**) (Girault et al., 1988).

Previously reported compounds from *C. vaga*

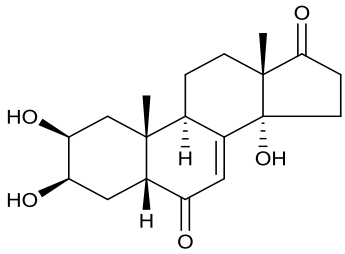
Previous phytochemical studies on *C. vaga* led to the isolation of two ecdysteroids, 20-hydroxyecdysone (**1**)(Santos et al., 1970) and Rubrosterone (**2**)(Chua et al., 1982), in addition to a macrocyclic hydrocarbon(Abela & Santos, 1974). Compounds (**1**) and (**2**) have been isolated in this study. HR-ESI-MS spectrum, and 1D NMR experiments, ^{1}H and ^{13}C, have been conducted and the results were compared with literature data.

First time reported compounds from *C. vaga*

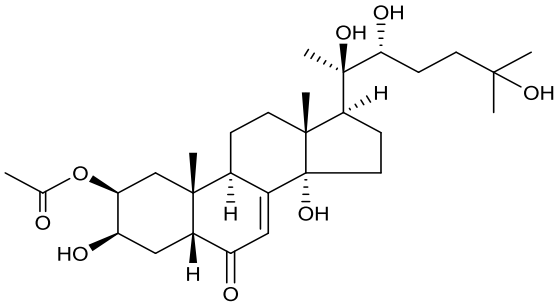
Even though compounds **3-8** are known compounds, this is the first time they are being reported from *C. vaga*. For their structure elucidations, ^{1}D and ^{2}D NMR experiments and HR-ESI-MS spectra have been established in order to gain enough knowledge about their structures then all data were compared with reported compounds data.



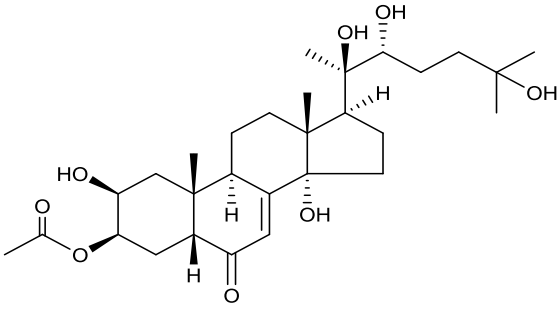
20-hydroxyecdysone (**1**)



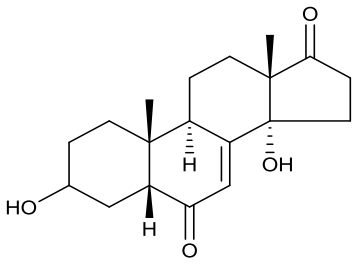
Rubrosterone (**2**)



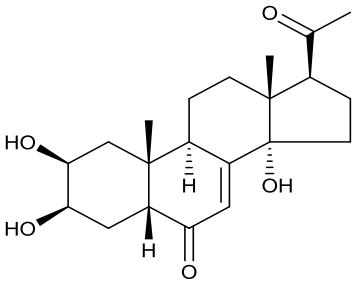
20-hydroxyecdysone 2-acetate(**3**)



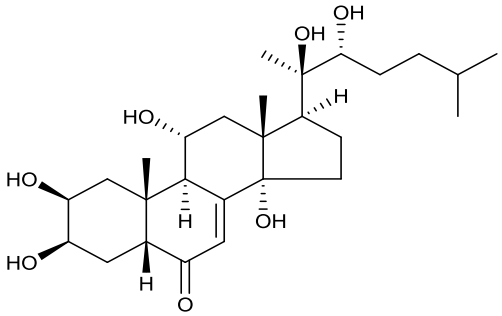
20-hydroxyecdysone 3-acetate(**4**)



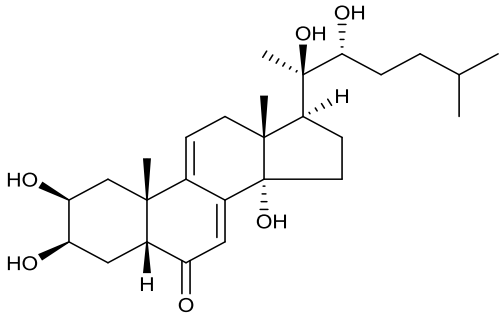
2-deoxyrubrosterone (**5**)



Poststerone (**6**)



Ajugasterone C (**7**)



Dacryhainansterone (**8**)

Figure 2-2 Structures of isolated compounds from *C. vaga*

Compound **1,** 20-hydroxyecdysone, was obtained as a major compound. The molecular formula was confirmed as $C_{27}H_{44}O_7$ based on the HR-ESI-MS spectrum(**Figure 2-3**) which gave an $[M+HCOO]^-$ ion at m/z 525.313 and an $[M+Cl]^-$ ion at m/z 515.284, in addition to an $[2M]^-$ ion at m/z 959.619 and $[2M+Cl]^-$ ion at m/z 995.596. Along with ^{13}C NMR (**Figure 2-4**) which shows 27 carbons resonances. ^1H NMR spectrum (**Figure 2-5**), and other compound's data are in agreement with the reported literature.

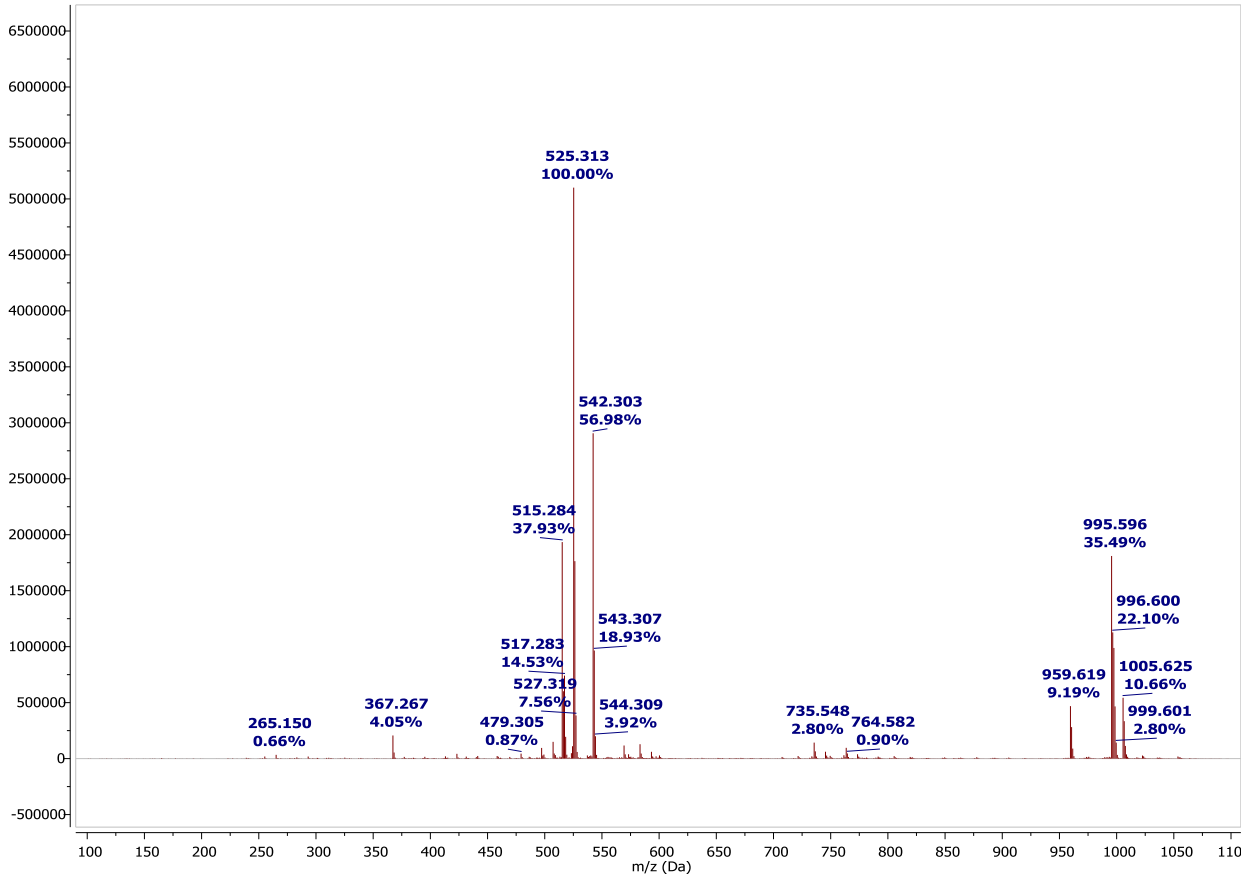


Figure 2-3 Mass spectrum of compound **1**

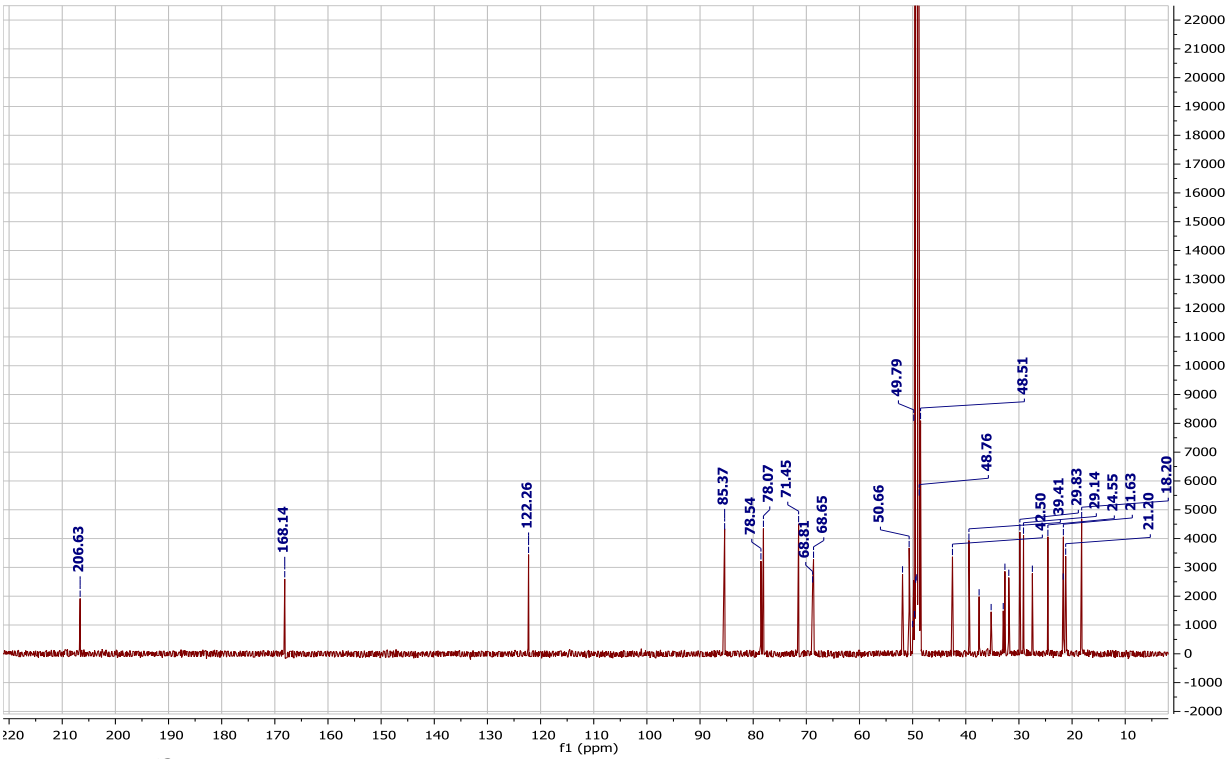


Figure 2-4 ^{13}C NMR spectrum of **1** in methanol-d_4

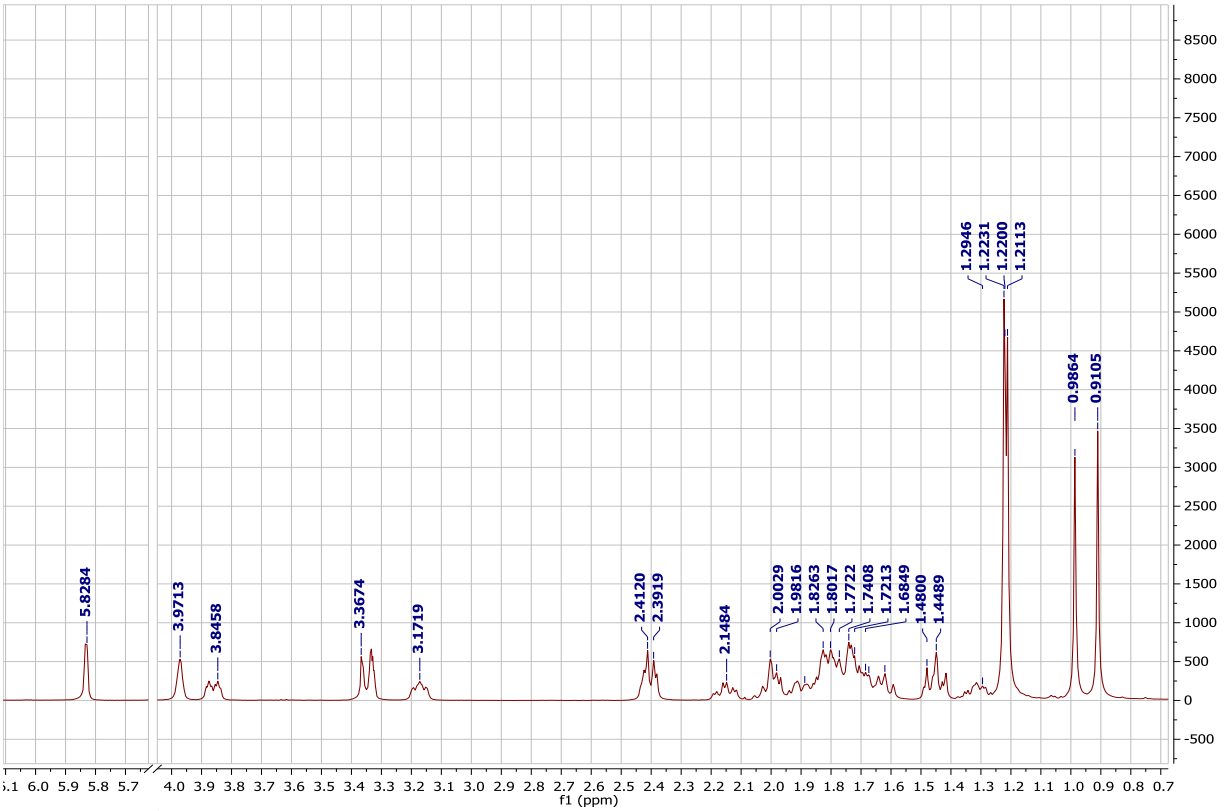


Figure 2-5 ^{1}H NMR spectrum of **1** in methanol-d_4

Compounds **2**, Rubrosterone, has been reported from this plant previously. It has a molecular formula of $C_{19}H_{26}O_{5,}$ which was determined based on ^{13}C NMR spectrum (**Figure 2-7**) and the HR-ESI-MS spectrum which gave deprotonated ions at m/z 335.187$[M+H]^+$, m/z 413.216$[M+DMSO]^+$, and 691.348$[2M+Na]^+$(**Figure 2-6**). From ^{1}H NMR (**Figure 2-8**), the characteristic proton of C-7 at 5.91 was detected. Protons 3.97(1H, d), and 3.85(1H, dt) represent hydroxylated carbons in compound **2**, beside C-14 which is dominated by a hydroxyl group in all isolated compounds. In accordance with DEPT-135, HSQC NMR spectra, the 19 carbons can be classified into two sp^3 methyls, six sp^3 methylenes, two sp^3 methines, two sp^3 oxygenated methines, an sp^2 methine, in addition to six non-protonated carbons.

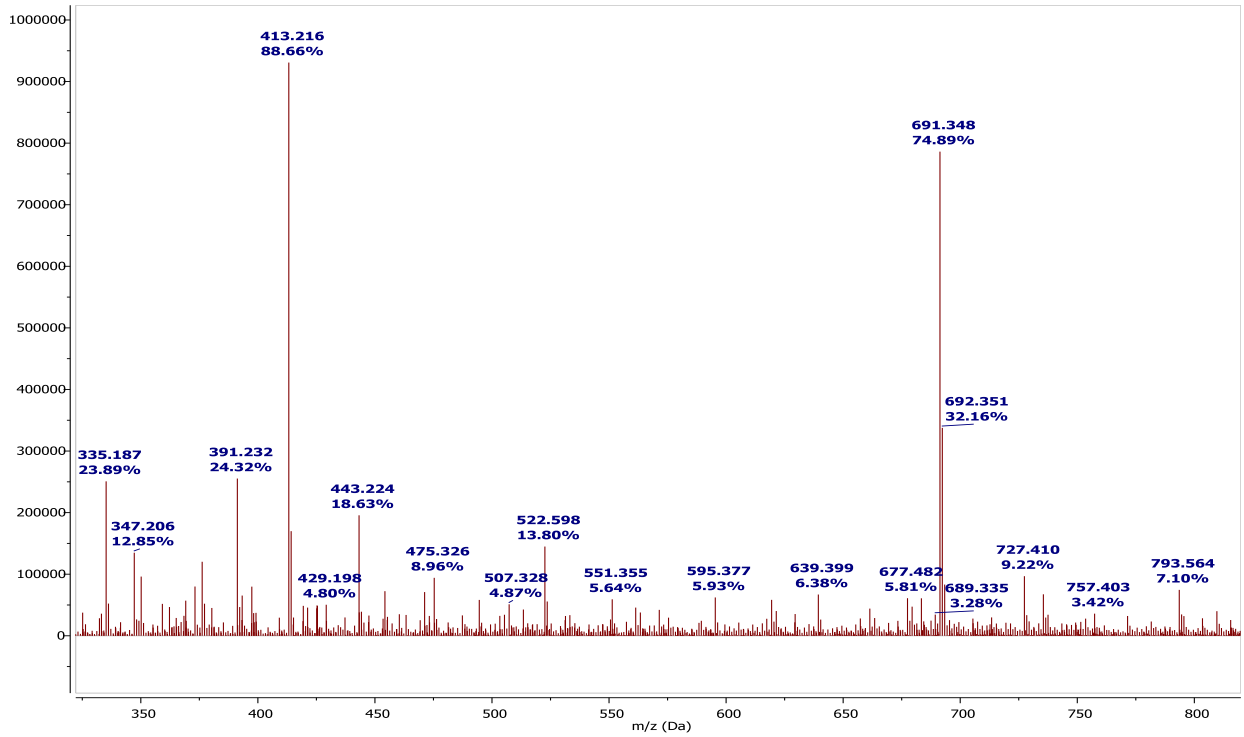


Figure 2-6 Mass spectrum of compound **2**

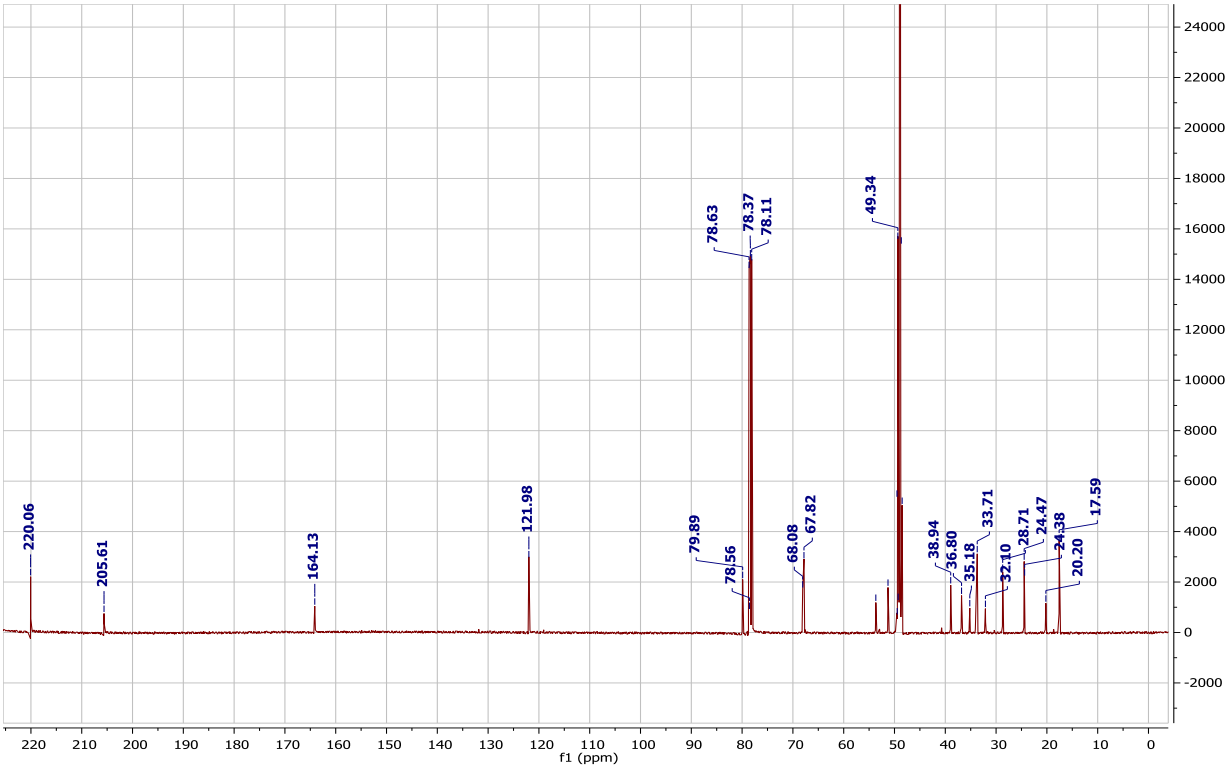


Figure 2-7 ^{13}C NMR spectrum of compound **2** in methanol-d_4/$CDCl_3$

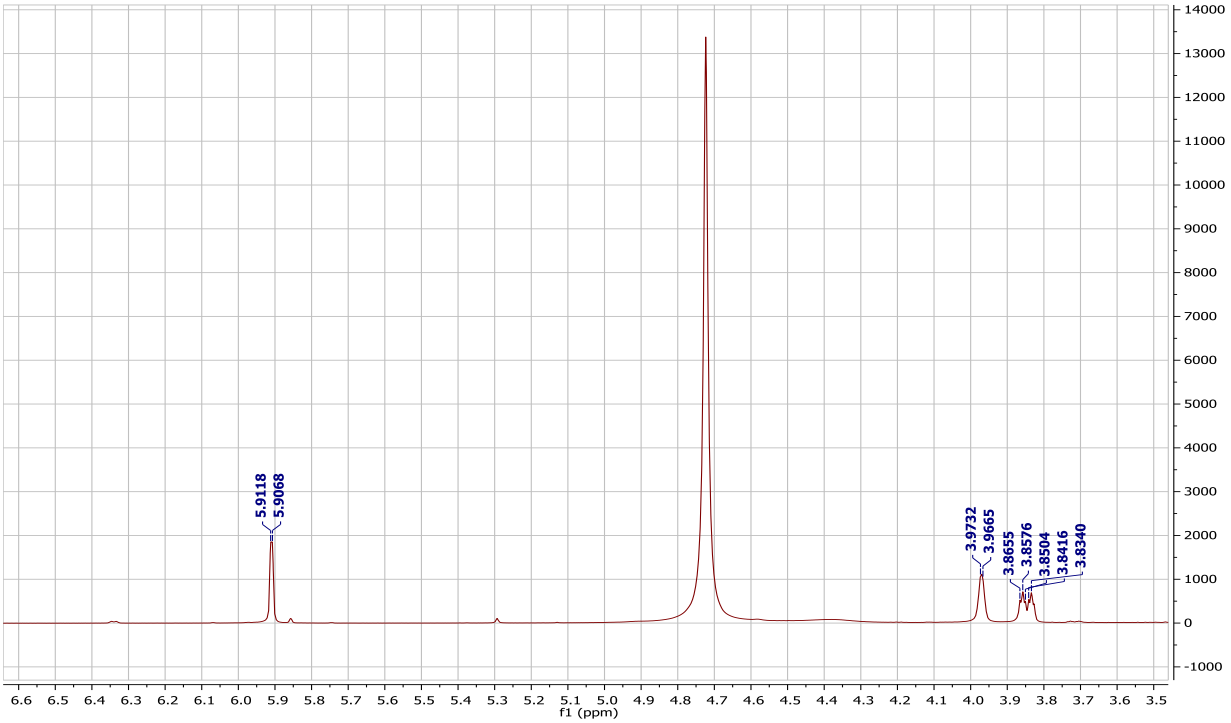


Figure 2-8 ^{1}H NMR spectrum of compound **2** in methanol-d_4/$CDCl_3$

Compound **3**, 20-hydroxyecdysone 2-acetate's, molecular formula was determined as $C_{29}H_{46}O_8$ from combined data of ^{13}C NMR (**Figure 2-9**) and protonated ions at m/z 545.310 $[M+Na]^+$ and 1067.629$[2M+Na]^+$ in the HR-ESI-MS (**Figure 2-10**)

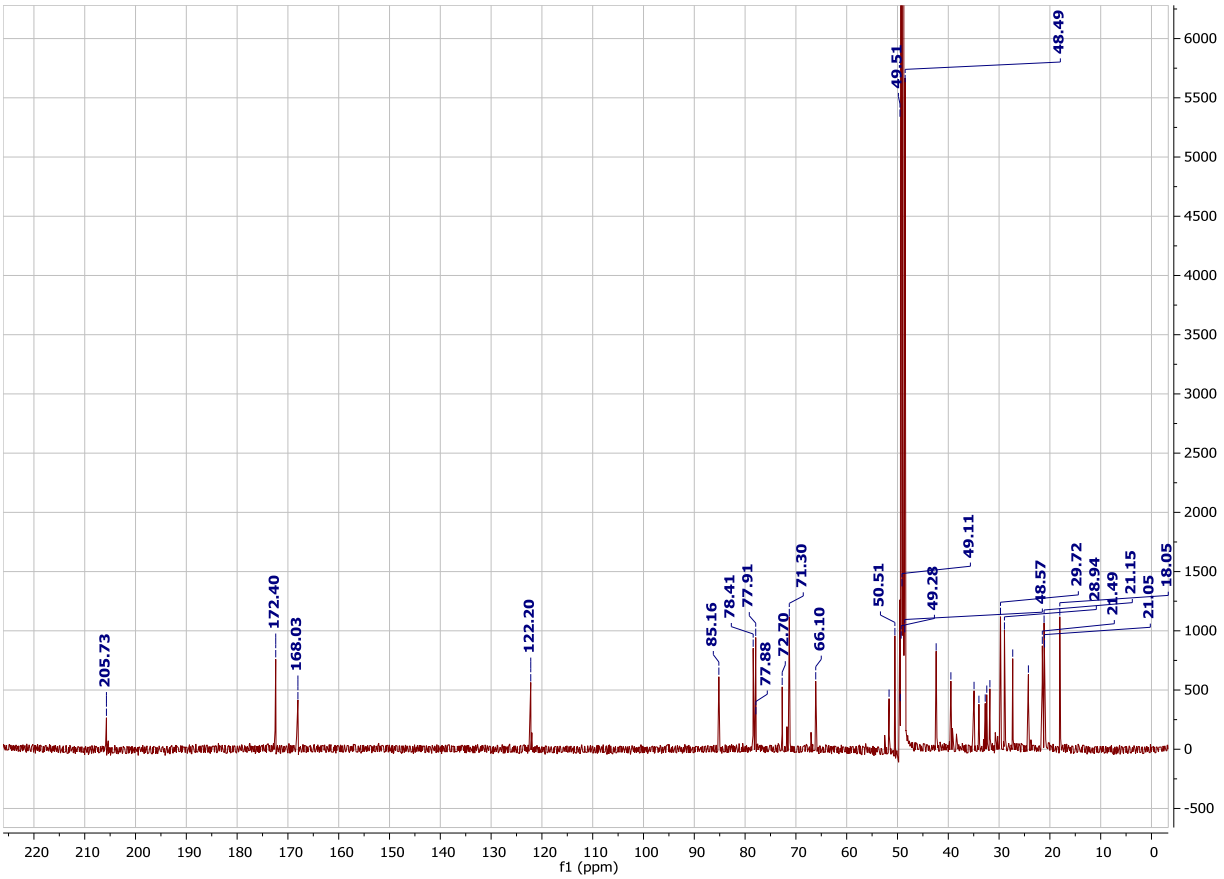


Figure 2-9 ^{13}C NMR spectrum of **3** in methanol-d_4

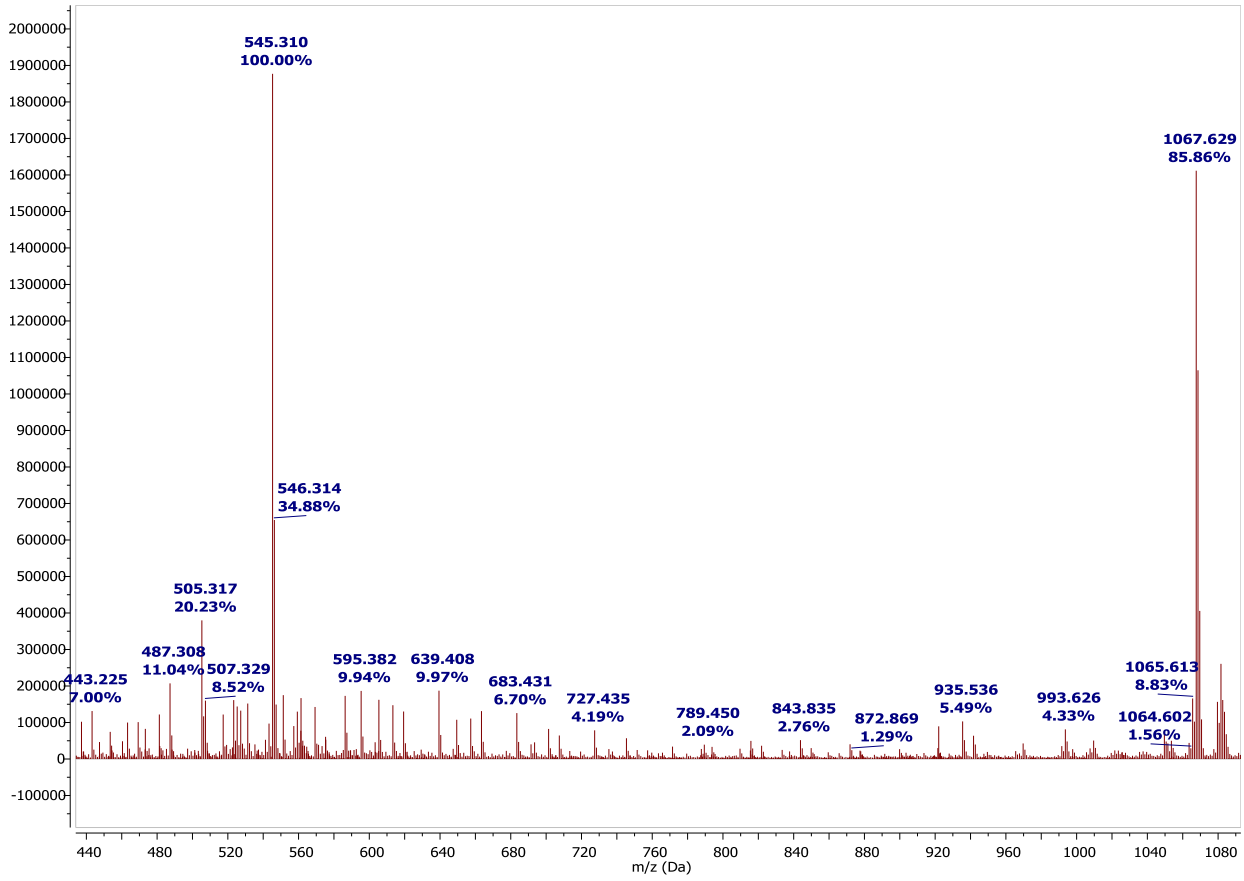


Figure 2-10 Mass spectrum of compound **3**

In ^{1}H NMR, the main difference feature than compound **1** is the singlet of (-OAc) at (2.08 ppm) which is a highly deshielded methyl group compared to the others (**Figure 2-11**).

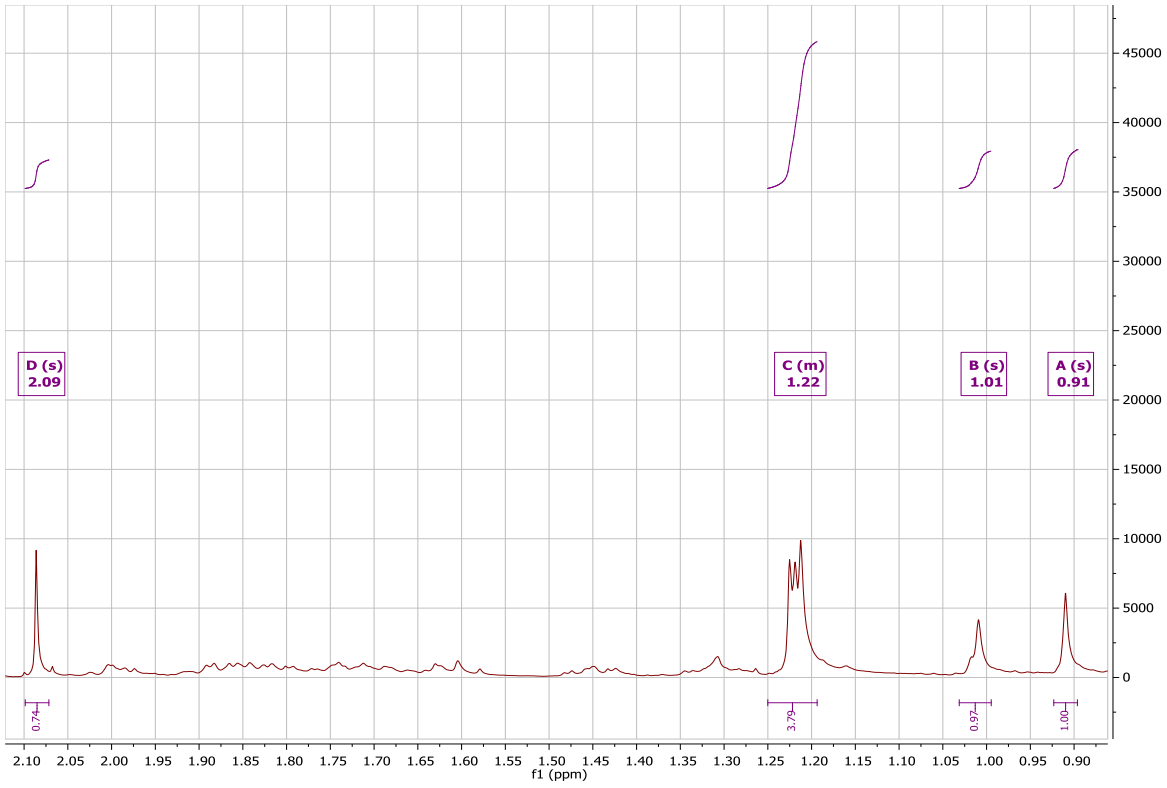


Figure 2-11 ^{1}H NMR spectrum of **3** in methanol-d_4

The carbon resonances (29 peaks) were classified into six sp^3 methyls, eight sp^3 methylenes, three sp^3 methines, three sp^3 oxygenated methines, an sp^2 methine, and seven non-protonated carbons based on ^{13}C, DEPT-135. Acetate position at C-2 was assigned in two steps, starting from matching 1D data of ^{1}H and ^{13}C through HSQC experiment (**Figure 2-12**) which then supported by the correlation of H-H neighboring coupling from COSY experiment (**Figure 2-13**) and that indicates two vicinal couplings between H-1α and H1β with H-2 which confirms the acetate's location at C-2. Also, from HMBC spectrum, there is a correlation that shows a long

range coupling between protons at position 19 and C-2 which also confirms C-2 acetate position (**Figure 2-14**).

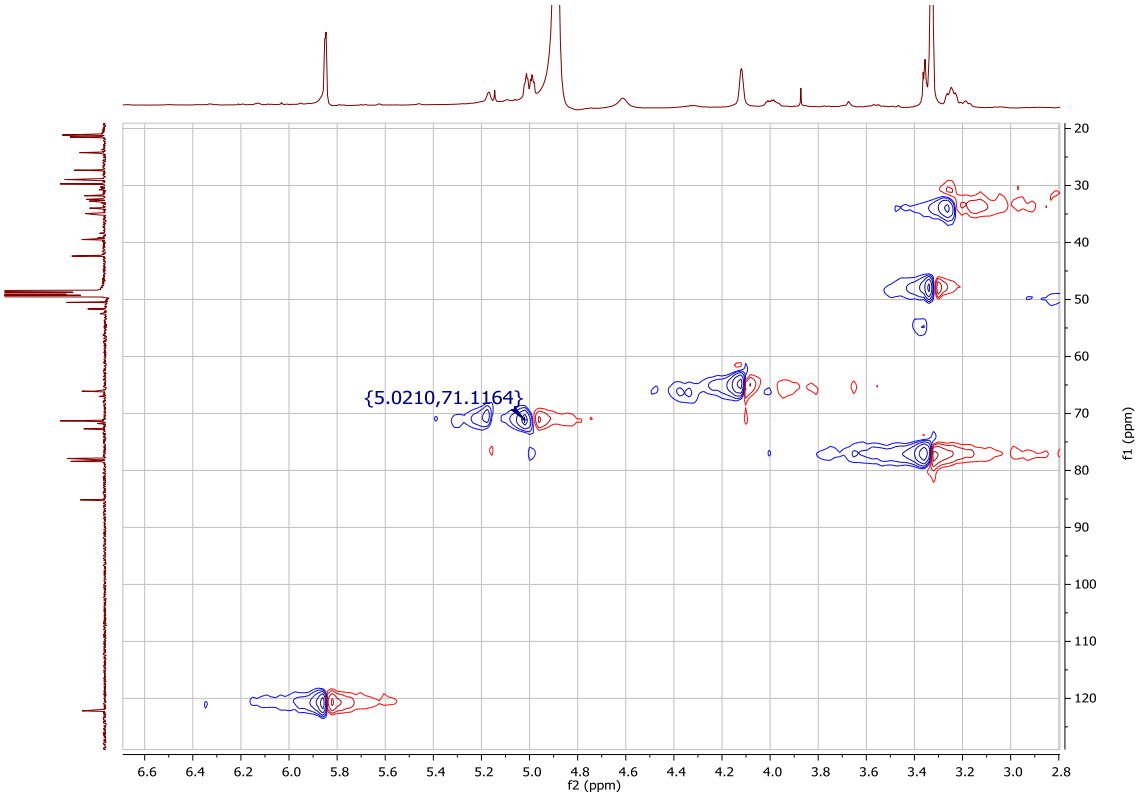


Figure 2-12 HSQC spectrum of compound **3** in methanol-d_4

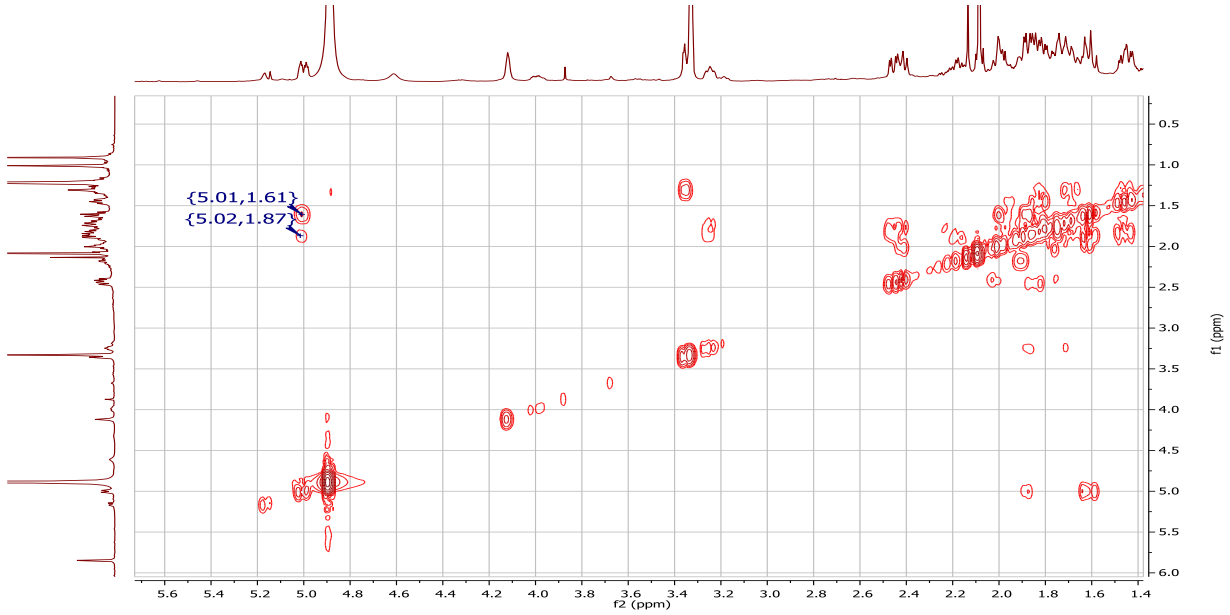


Figure 2-13 COSY spectrum of compound **3** in methanol-d_4

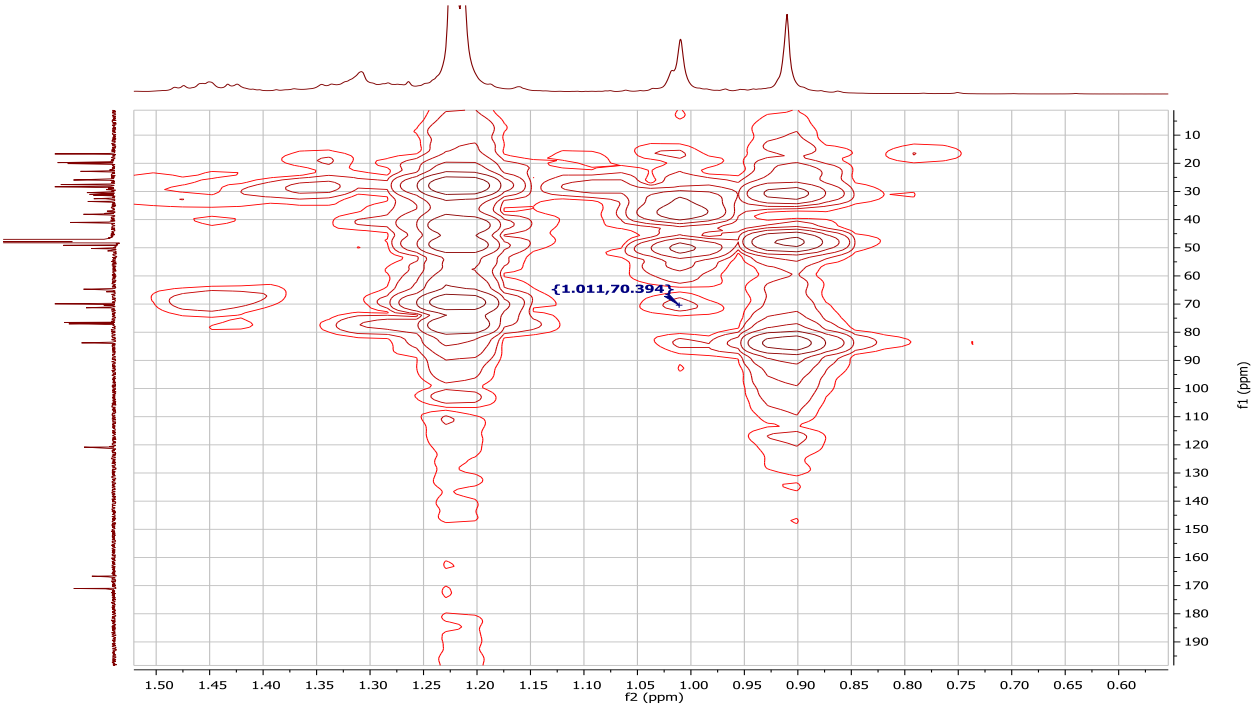


Figure 2-14 HMBC spectrum of compound **3** in methanol-d_4

Compound **4**, 20-hydroxyecdysone 3-acetate, is also an acetate derivative of 20-hydroxyecdysone, has the molecular formula of $C_{29}H_{46}O_{8,}$ based on ^{13}C NMR, 29 carbons resonances (**Figure 2-15, Figure 2-16**) and HR-ESI-MS spectrum which gave protonated ions at m/z 523,327$[M+H]^+$, 545.309 $[M+Na]^+$, 561.283 $[M+K]^+$ and 1067.629 $[2M+Na]^+$ (**Figure 2-17)**.

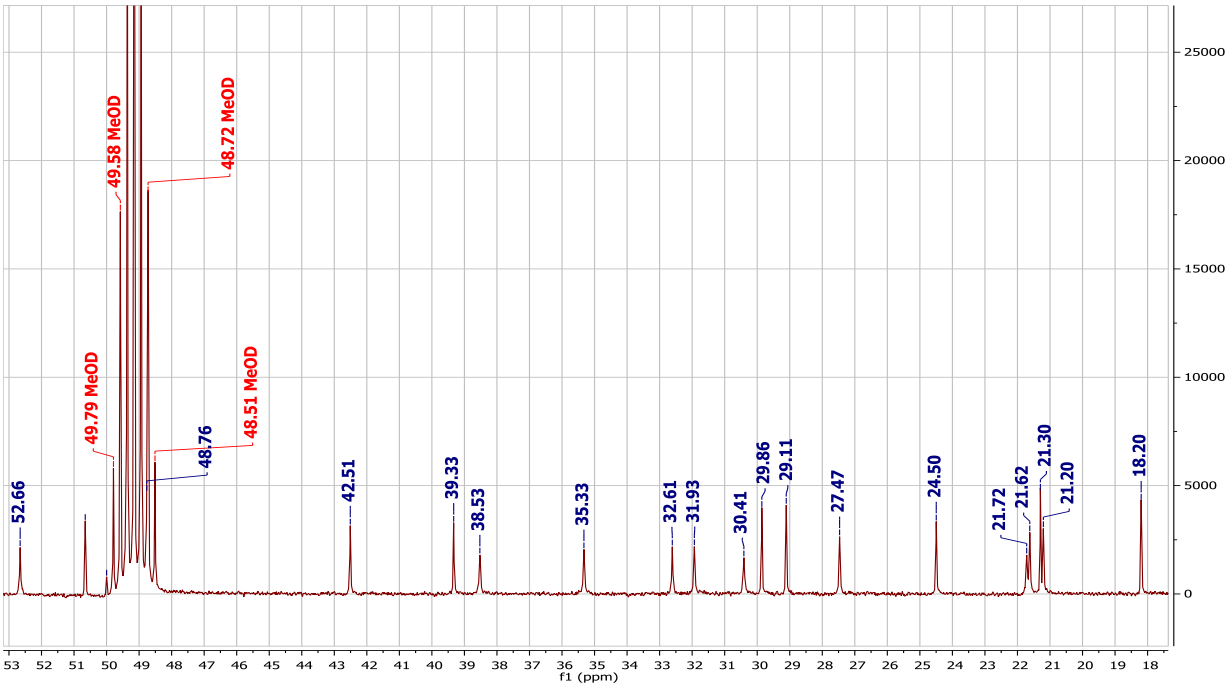


Figure 2-15 ^{13}C NMR spectrum-A of **4** in methanol-d_4

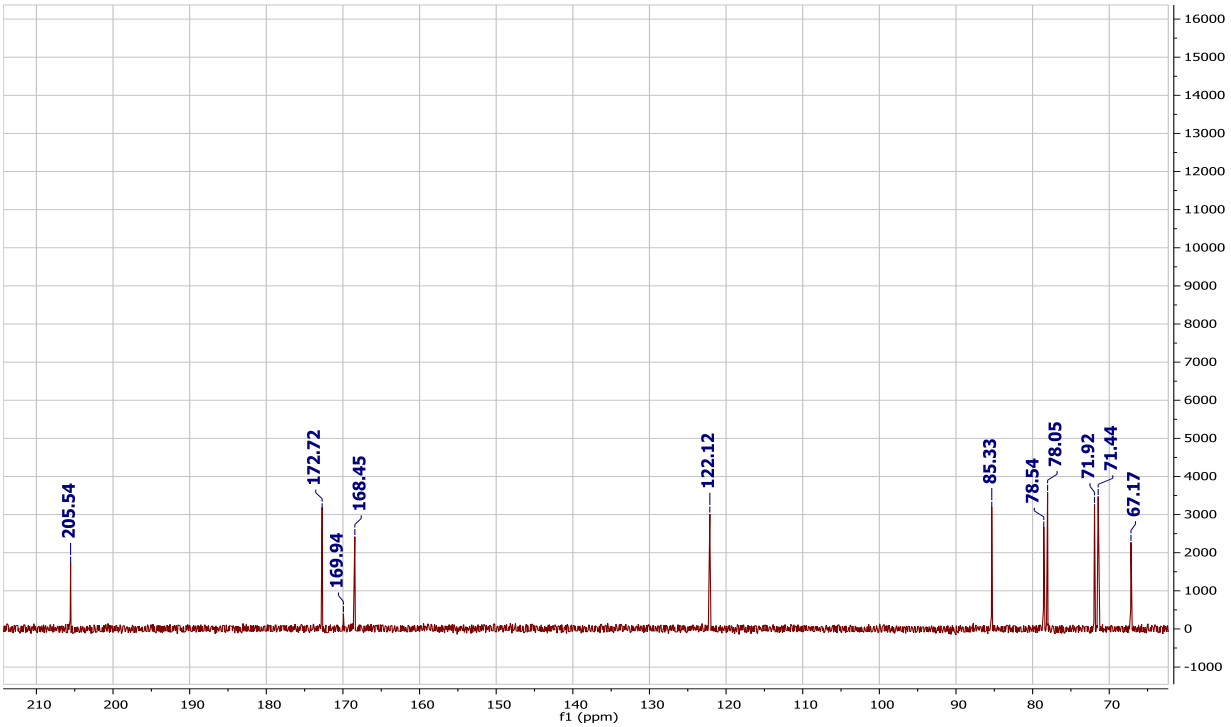


Figure 2-16 ^{13}C NMR spectrum-B of compound **4** in methanol-d_4

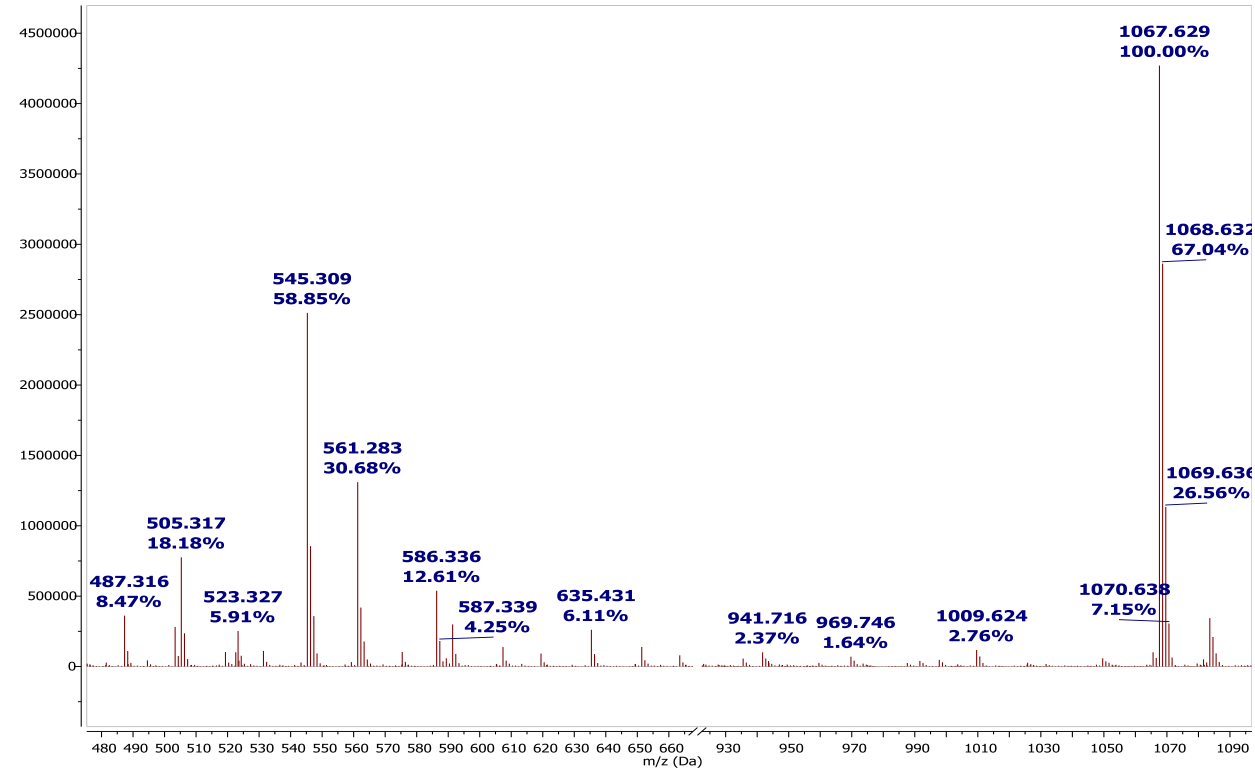


Figure 2-17 Mass spectrum of compound **4**

^{1}H NMR of compound **4** shows protons 2 and 3 at (4.00 ppm, 5.17 ppm) respectively (**Figure 2-18**), followed by HSQC to correlate them with corresponding carbons (**Figure 2-19**), which indicates that position-3 is acetylated.

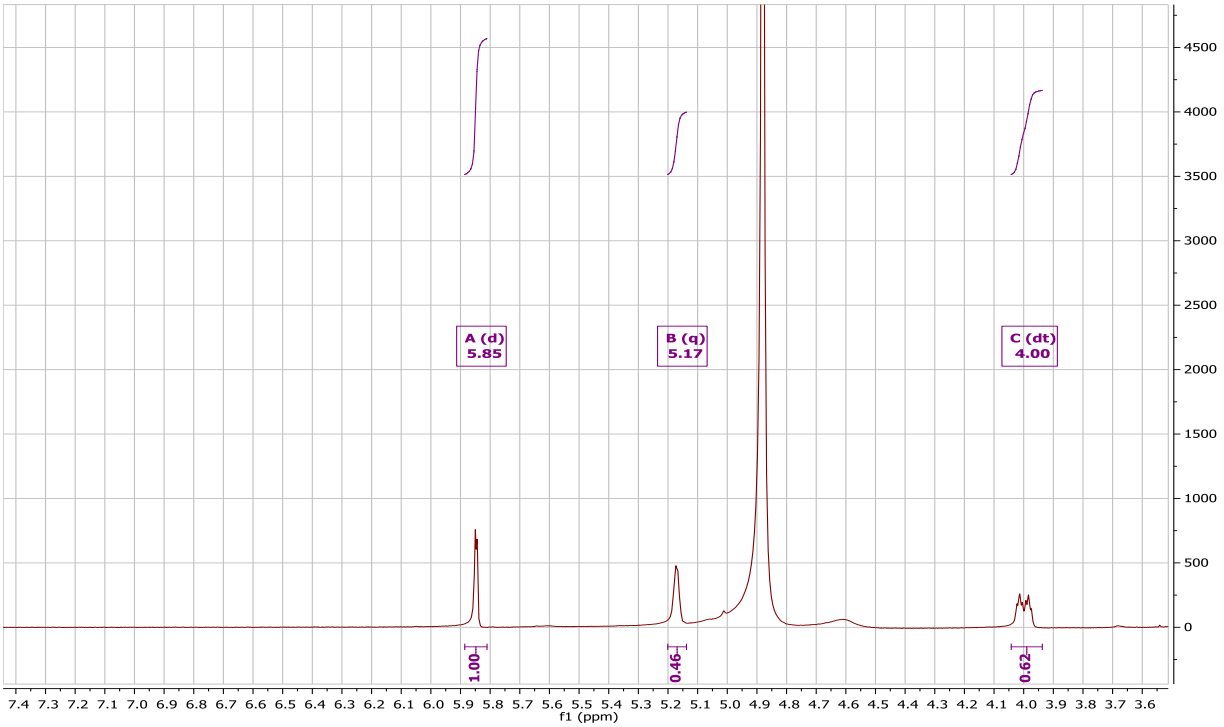


Figure 2-18 ^{1}H NMR spectrum of compound **4** in methanol-d_4

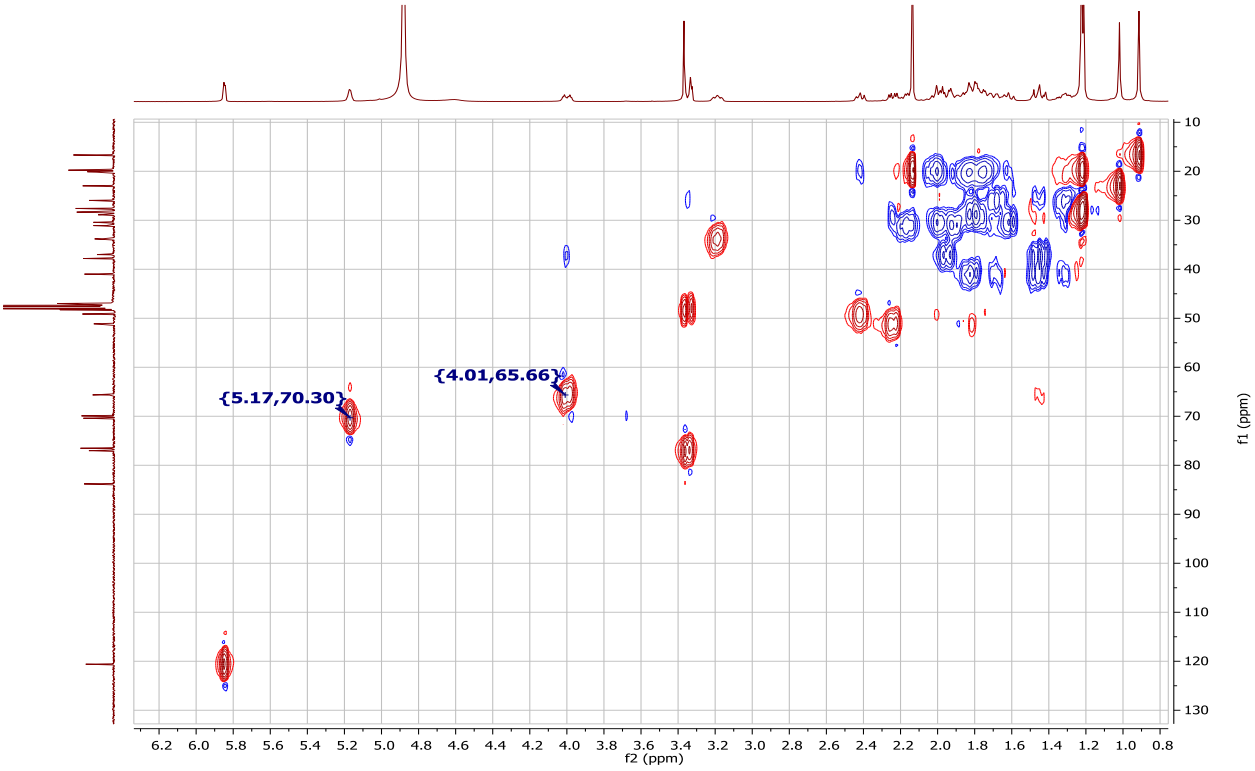


Figure 2-19 HSQC spectrum of compound **4** in methanol-d_4

Compound **5**, 2-deoxyrubrosterone, was obtained as a minor compound with very low yield (2.2mg). The molecular formula was determined as $C_{19}H_{26}O_4$ according to DEPTQ-135 NMR spectrum (**Figure 2-20**), and the HR-ESI-MS spectrum gave deprotonated ions at m/z 353.157 $[M+Cl]^-$, and 363.186 $[M+HCOO]^-$ (**Figure 2-21**). 2-deoxyrubrosterone is similar to compound **2,** rubrosterone, except that it has only two hydroxyl groups instead of three. In the HSQC experiment, only one oxygenated methine carbon appears at 70.08 ppm, along with a methine at 53.81, position 5, correlating with 3.57 and 2.41 respectively (**Figure 2-22**).

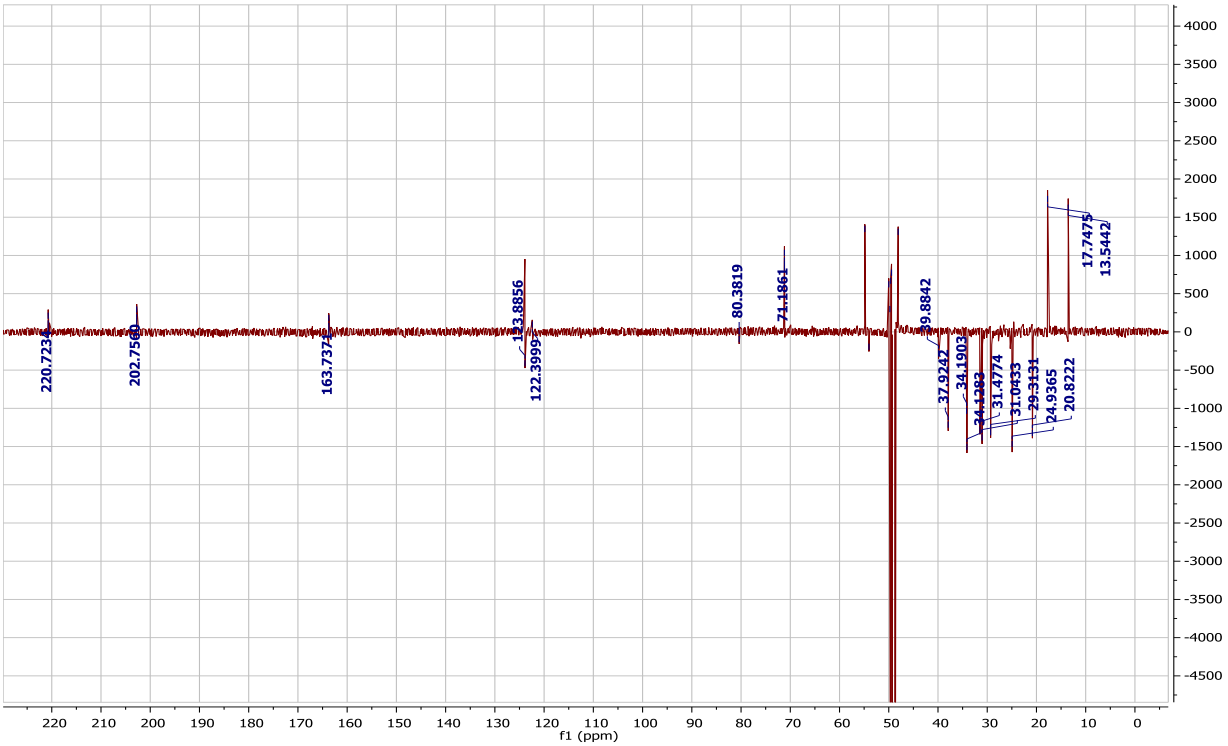


Figure 2-20 DEPTq-135 spectrum of compound **5** in methanol-d_4

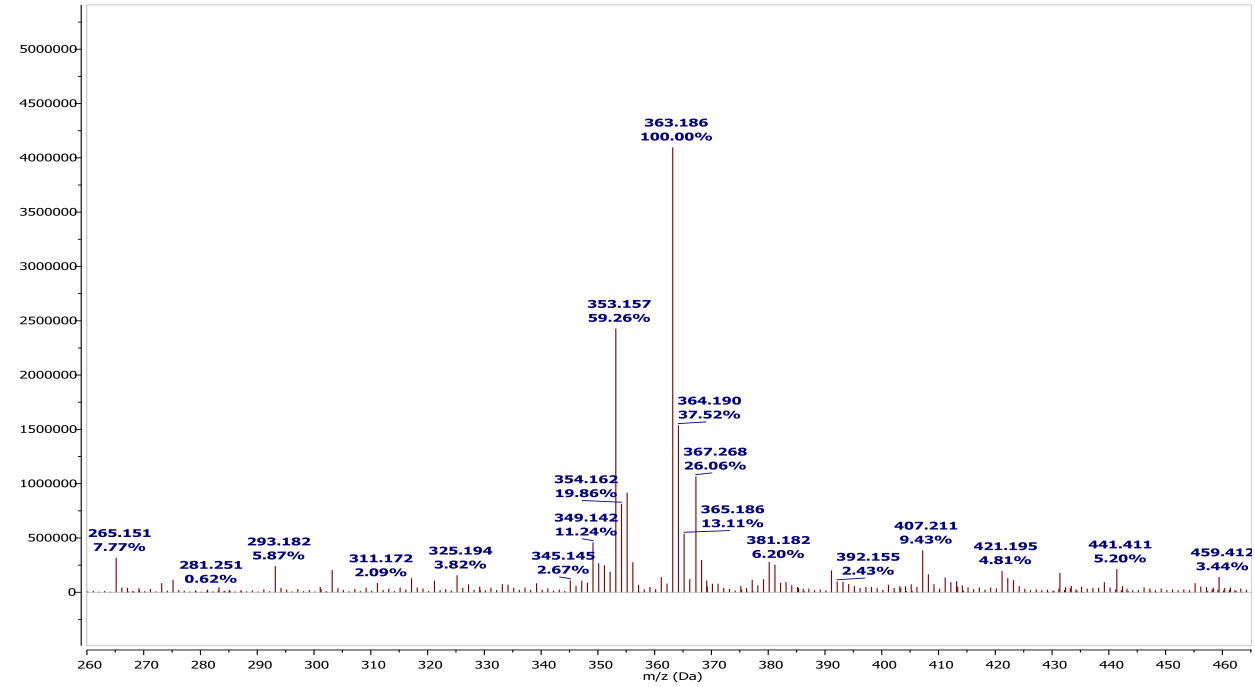


Figure 2-21 Mass spectrum of compound **5**

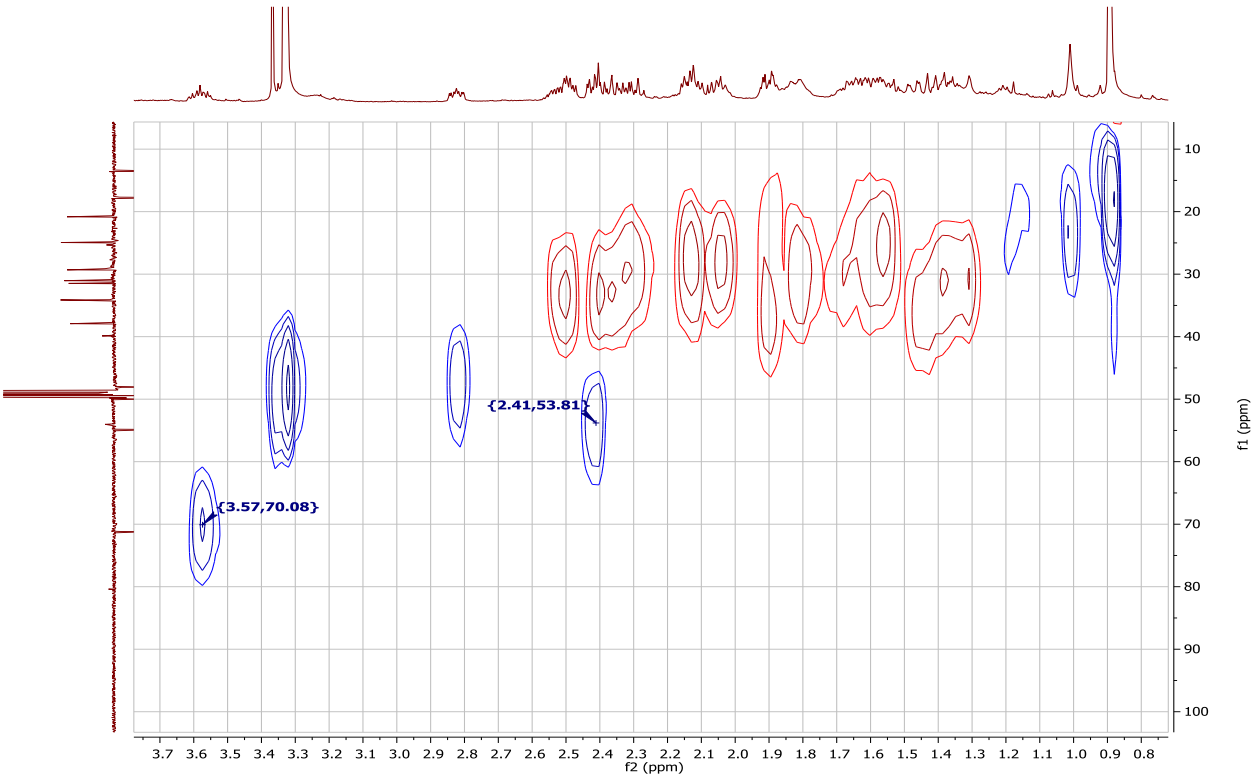


Figure 2-22 HSQC spectrum of compound **5** in methanol-d_4

Poststerone, compound **6**, is a 21 carbon ecdysteroids. The molecular formula, $C_{21}H_{30}O_5$, was determined based on the ^{13}C NMR spectrum(**Figure 2-23**), and the HR-ESI-MS spectrum which showed deprotonated ions at m/z 397.184 $[M+Cl]^-$, and at m/z 407.212 $[M+HCOO]^-$ (**Figure 2-25**).

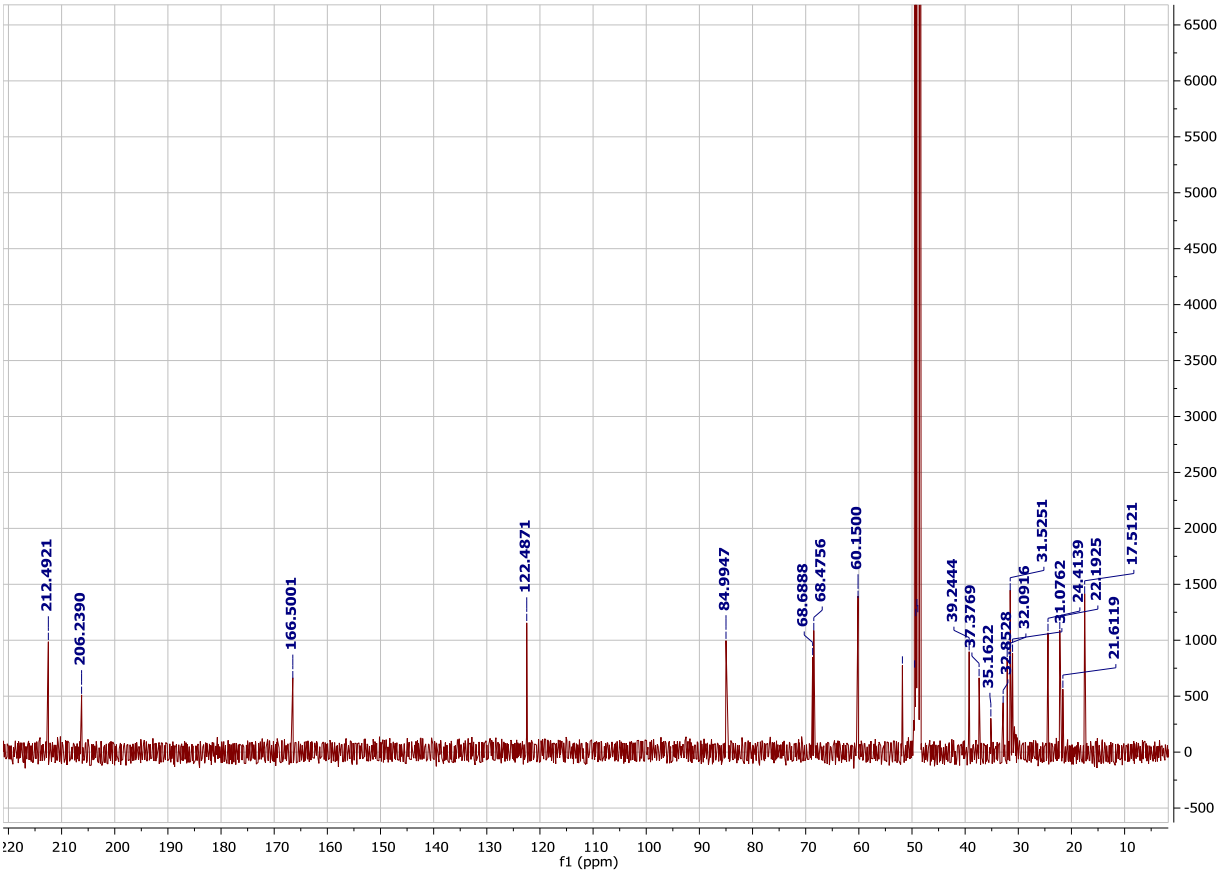


Figure 2-23 ^{13}C NMR spectrum of compound **6** in methanol-d_4

The ^1H NMR spectrum shows a characteristic methyl group singlet of acetyl functional group 2.17 (3H) (**Figure 2-24**), which also has been detected in compound 2 and 3 as they both possess acetyl functional groups in different positions.

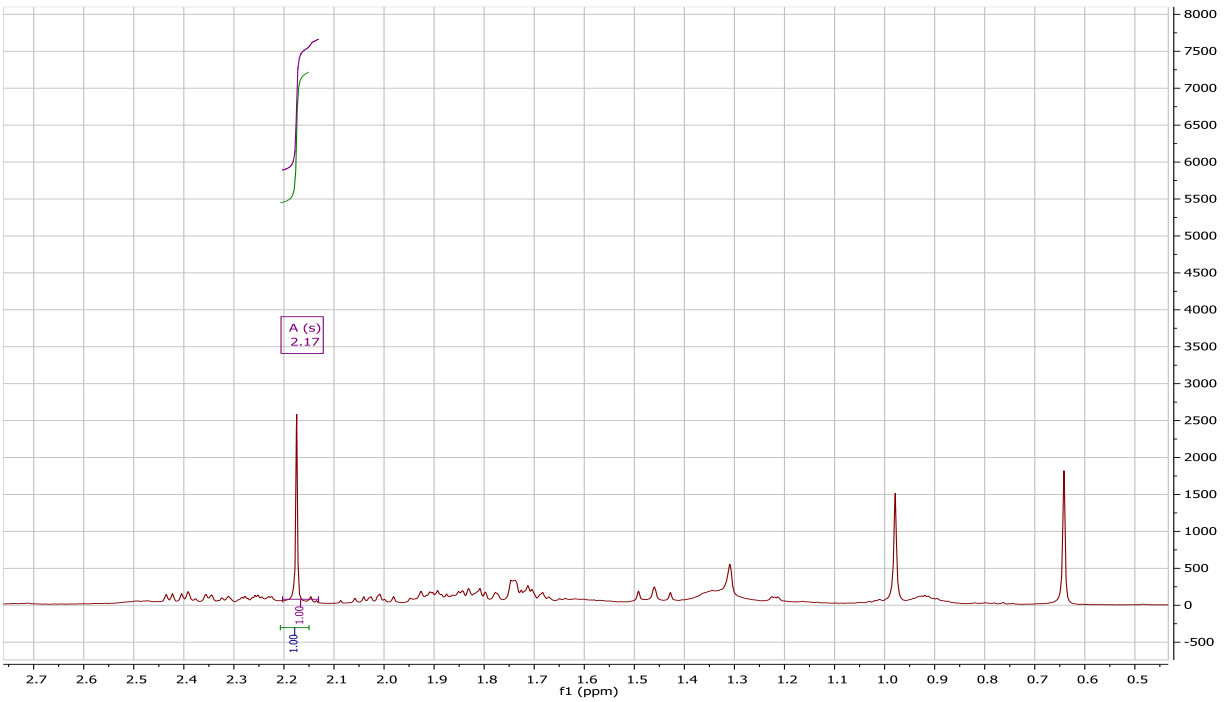


Figure 2-24 ^{1}H NMR spectrum of compound **6** in methanol-d_4

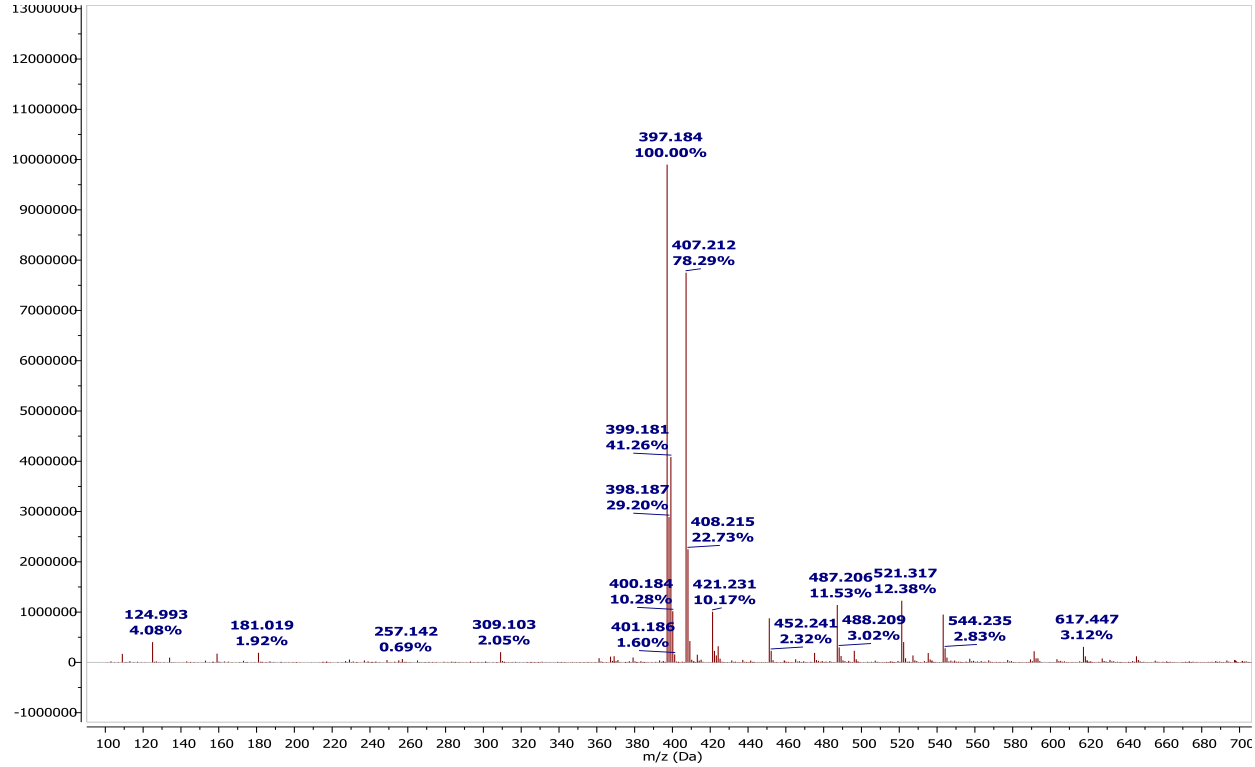


Figure 2-25 Mass spectrum of compound **6**

The 21 carbons resonances of compound **6** in ^{13}C NMR spectrum were classified by support of DEPT-135 and HSQC NMR data into three sp^3 methyls, six sp^3 methylene, three sp^3 methines, two oxygenated methines, an sp^2 methine, and six non-protonated carbons **(Figure 2-26, Figure 2-27**).

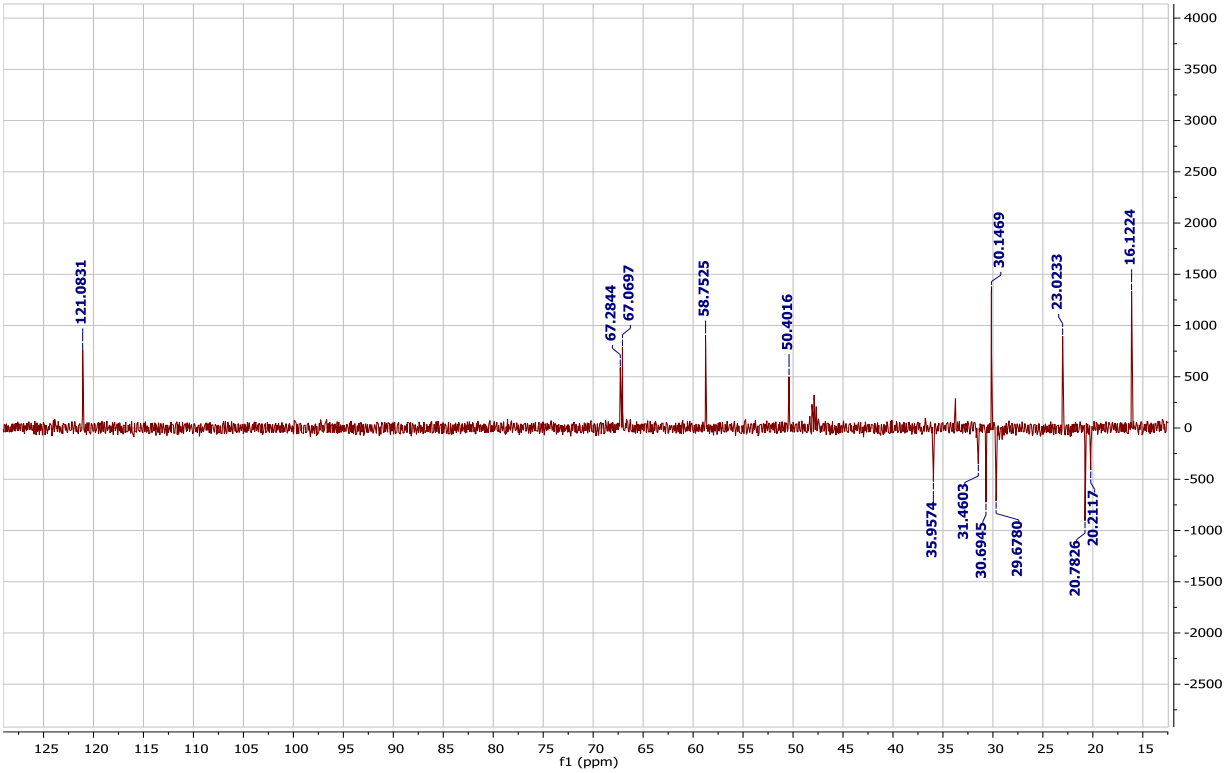


Figure 2-26 DEPT-135 spectrum of compound **6** in methanol-d_4

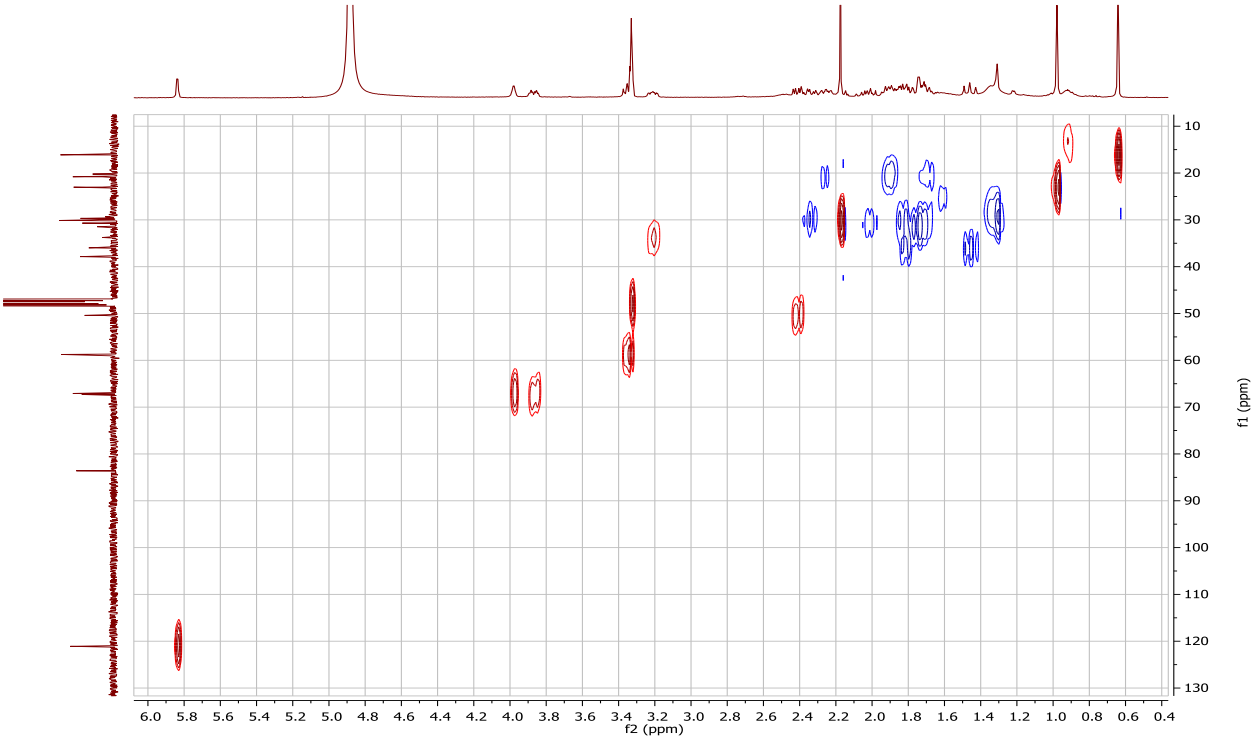


Figure 2-27 HSQC-NMR spectrum of compound **6** in methanol-d_4

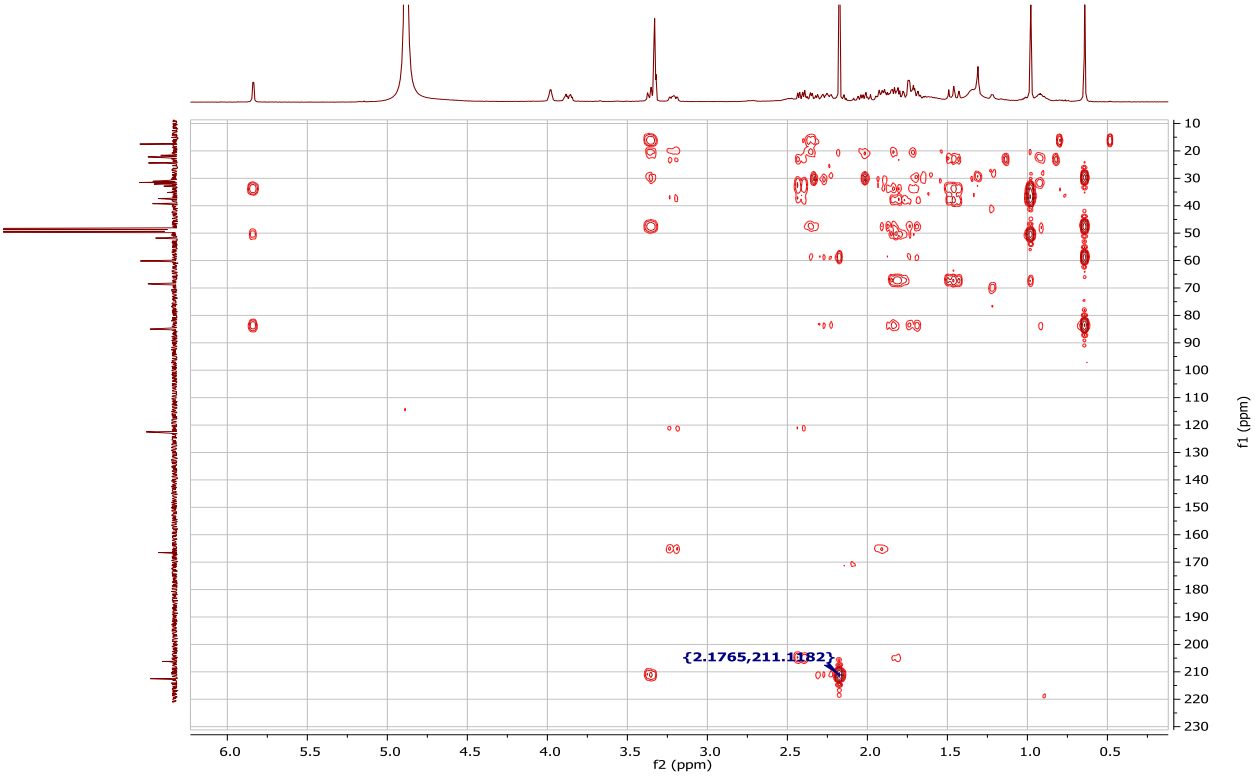


Figure 2-28 HMBC-NMR spectrum of compound **6** in methanol-d_4

Compound 7, ajugasterone C, is the second major compound which also has 27 carbons and the same molecular formula of compound **1**(20-hydroxyecdysone), $C_{27}H_{44}O_{7.}$ The molecular formula was obtained based on ^{13}C NMR data of the molecule (**Figure 2-30**) and the HR-ESI-MS spectrum which gave an $[M+HCOO]^-$ ion at m/z 525.312 and an $[M+Cl]^-$ ion at m/z 515.283, in addition to an $[2M+H]^-$ ion at m/z 959.617, $[2M+Cl]^-$ ion at m/z 995.594, and $[2M+HCOO]^-$ ion at m/z1005.623 (**Figure 2-31**). The only difference between compound **1** and **7** is one hydroxyl group; In compound **1**, C-25 is hydroxylated while C-11 is in compound **7**. Protons 26 and 27 usually appear as singlets due to the hydroxylation of C25; however, in compound **7**, they show doublets as they coupled with proton 25 since it is not hydroxylated **(Figure 2-29)**. 2D-NMR experiments HSQC (**Figure 2-34**), COSY (**Figure 2-33**), and HMBC clarify and support some overlapped data in the 1D-NMR spectra.

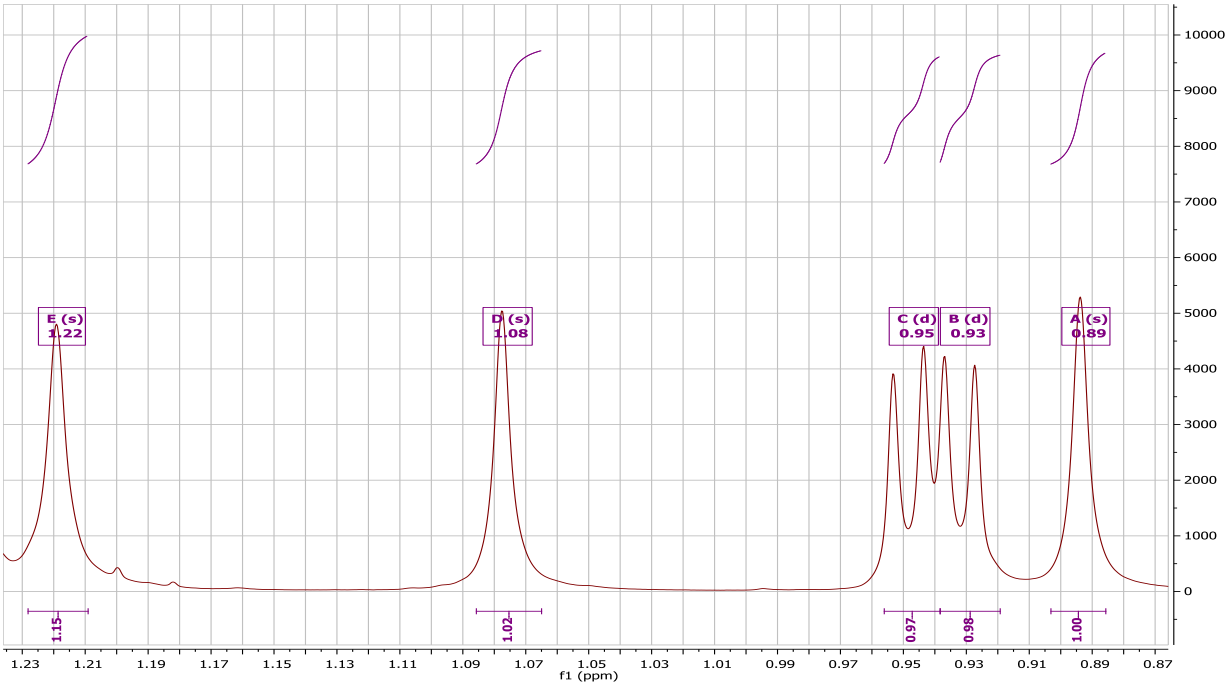


Figure 2-29 ^1H NMR spectrum of compound **7** in methanol-d_4

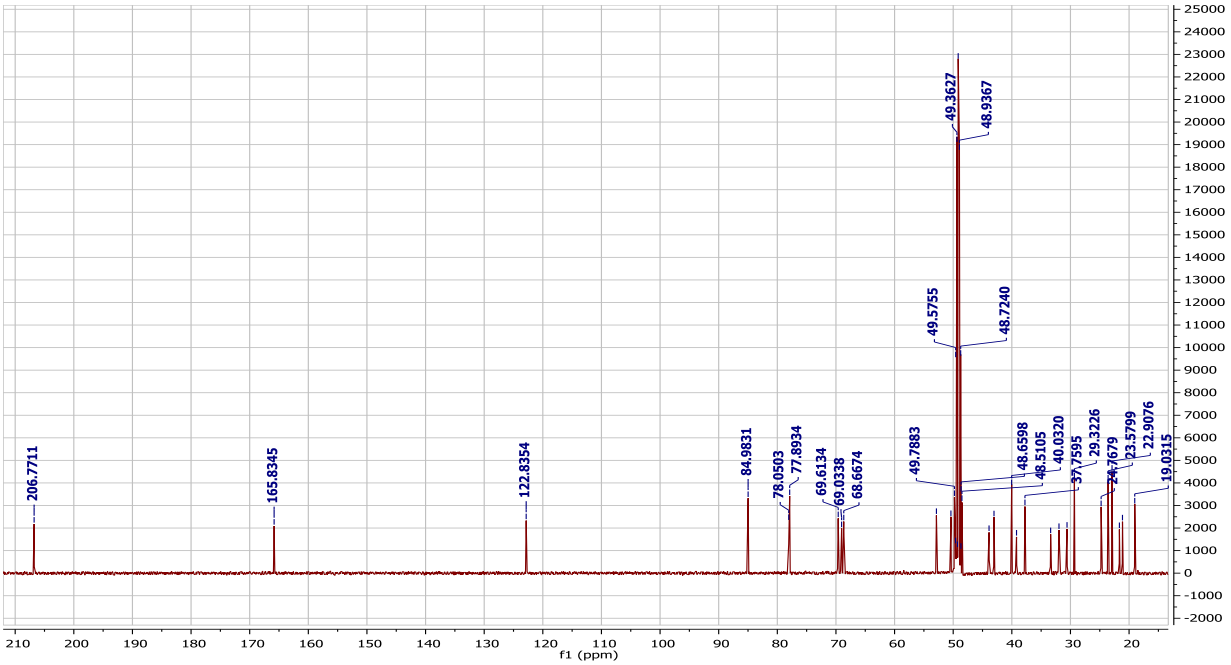


Figure 2-30 ^{13}C NMR spectrum of compound **7** in methanol-d_4

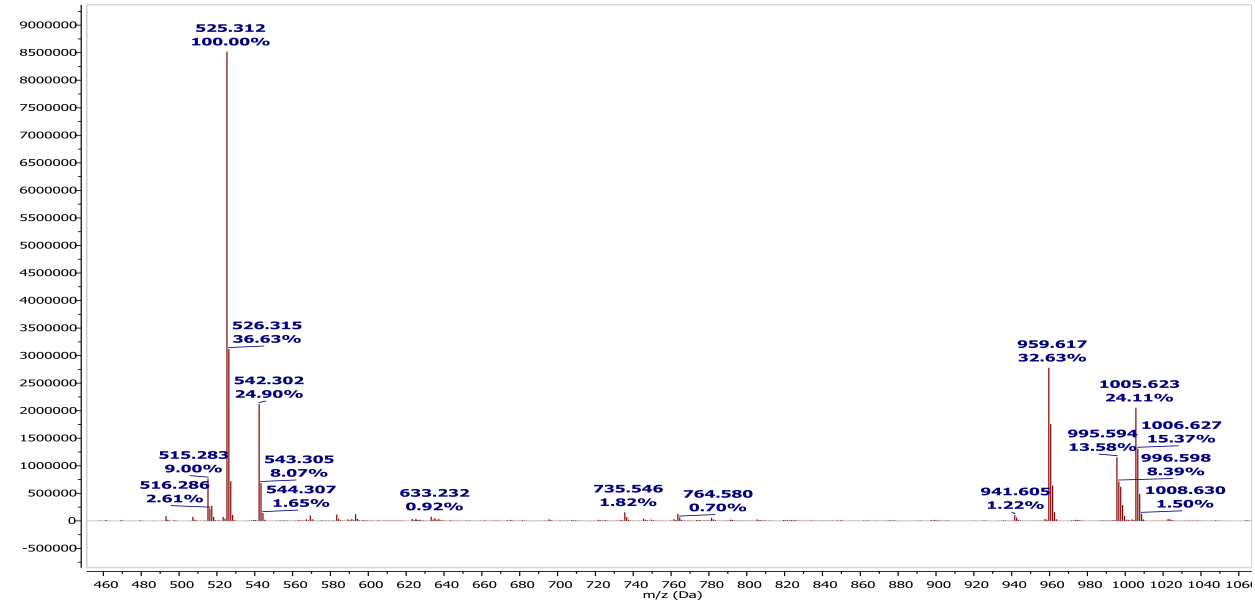


Figure 2-31 Mass spectrum of compound **7**

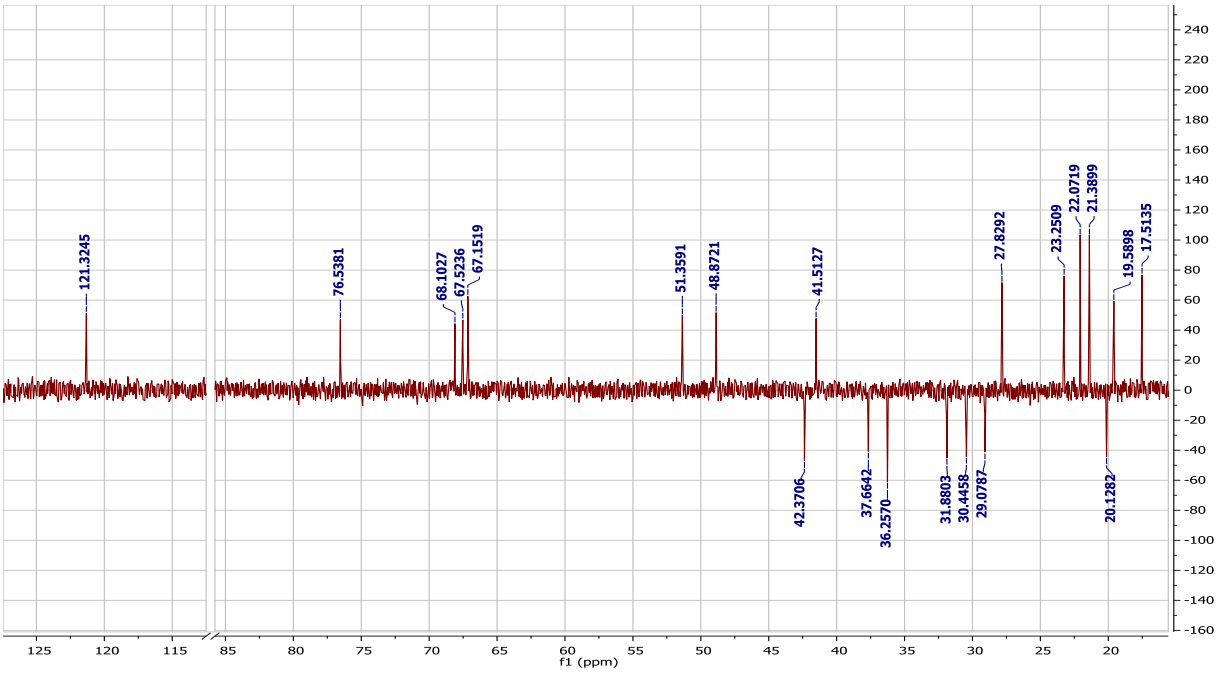


Figure 2-32 DEPT-135- NMR spectrum of compound **7** in methanol-d_4

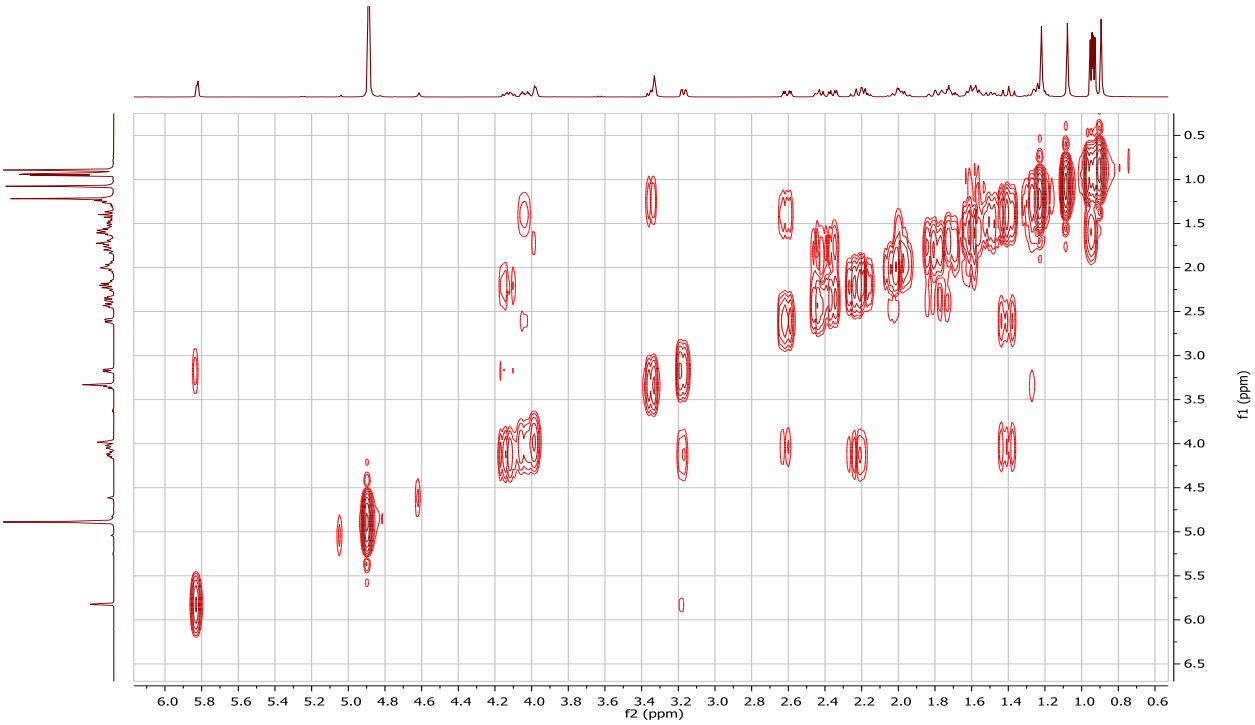


Figure 2-33 COSY- NMR spectrum of compound **7** in methanol-d_4

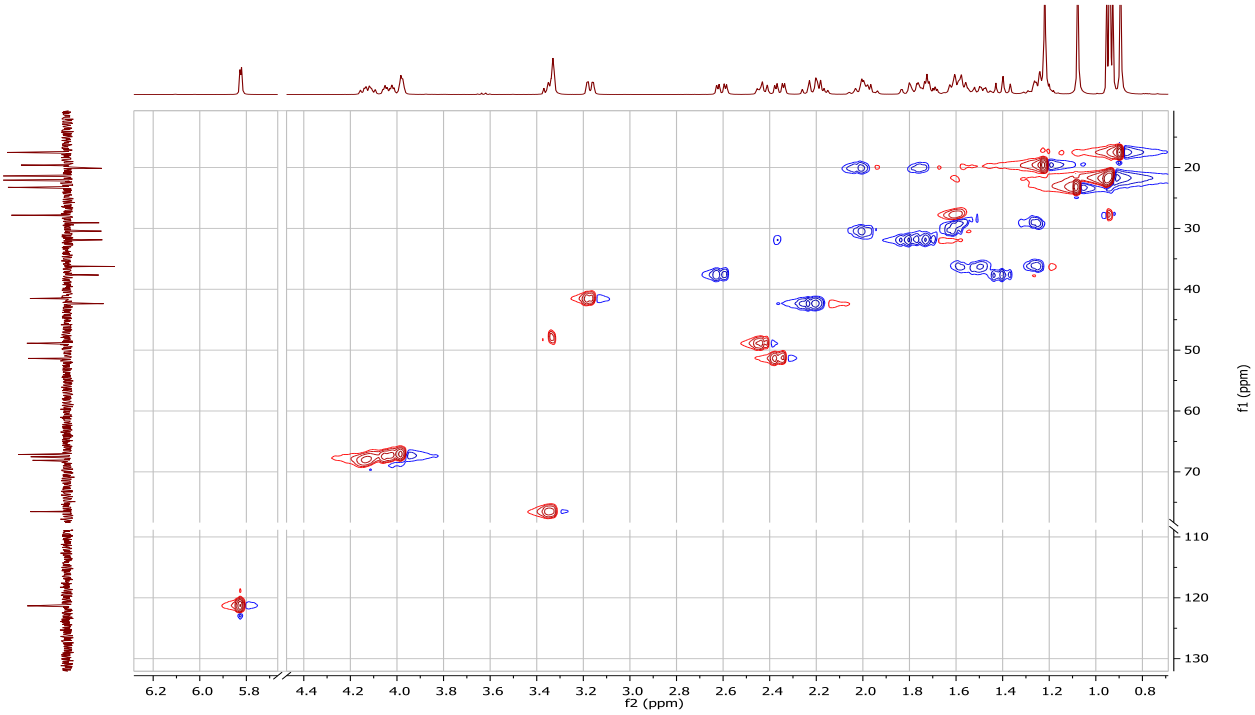


Figure 2-34 HSQC- NMR spectrum of compound **7** in methanol-d_4

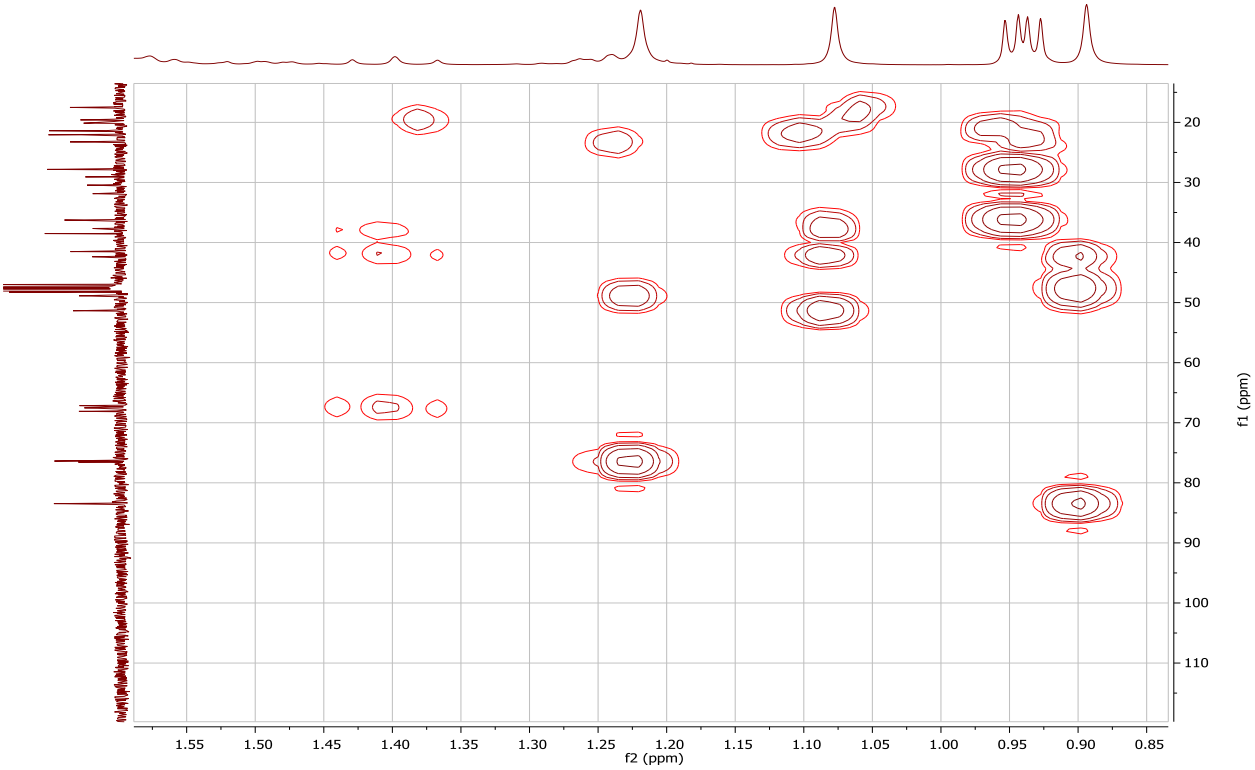


Figure 2-35 HMBC-NMR spectrum of compound **7** in methanol-d_4

Compound 8, Dacryhainansterone, is the only compound which has two double bonds among the isolated compounds. The molecular formula was obtained based on ^{13}C NMR data of the molecule (**Figure 2-37**) and the HR-ESI-MS spectrum which gave a $[M+H]^+$ ion at m/z 463.306 (**Figure 2-38**). Proton NMR shows the same pattern of doublets for 26 and 27 due to their couplings with proton 25. Moreover, proton 18 overlapped with them (**Figure 2-36**). DEPT-135 spectrum shows 20 resonances for all compound's carbons except seven non-protonated carbons (**Figure 2-39**). 2D-NMR experiments HSQC (**Figure 2-40**), COSY (**Figure 2-41**), and HMBC (**Figure 2-42**) clarify and support some overlapped data in 1D-NMR.

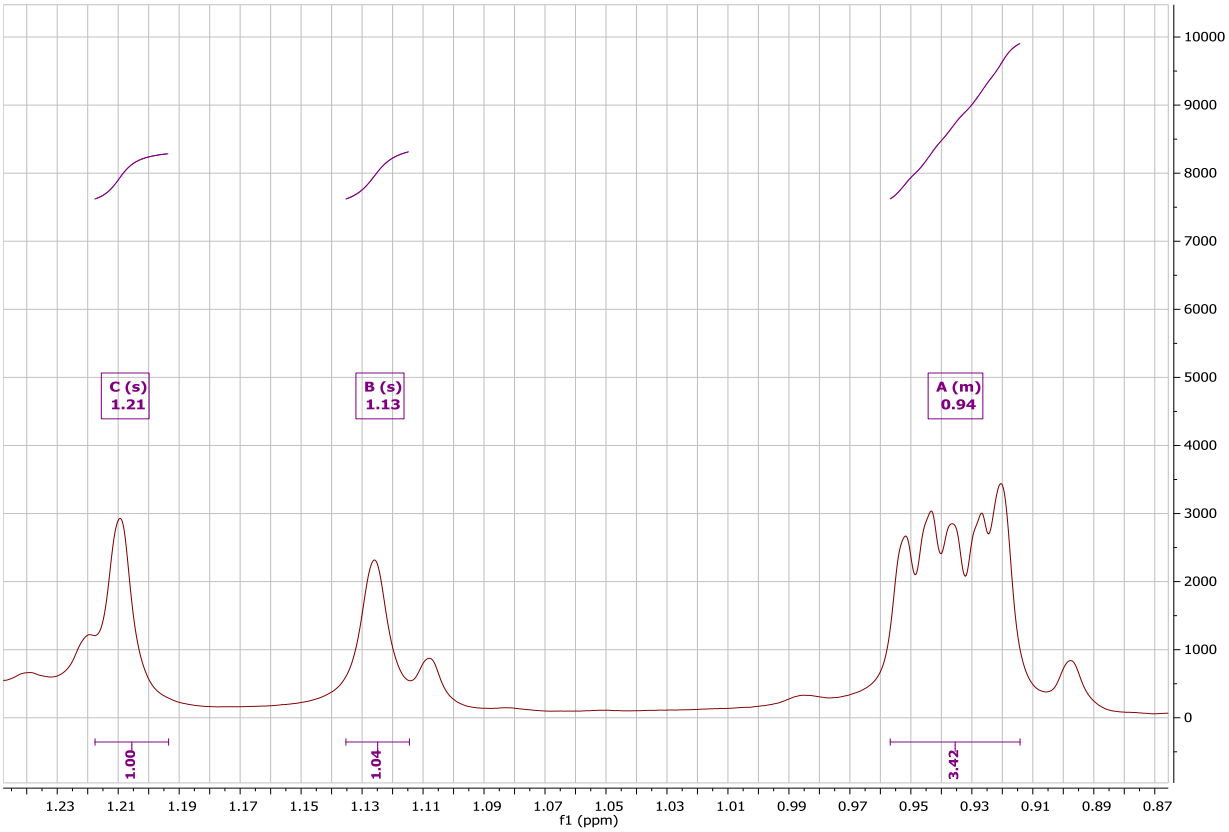


Figure 2-36 ^1H NMR spectrum of compound **8** in methanol-d_4

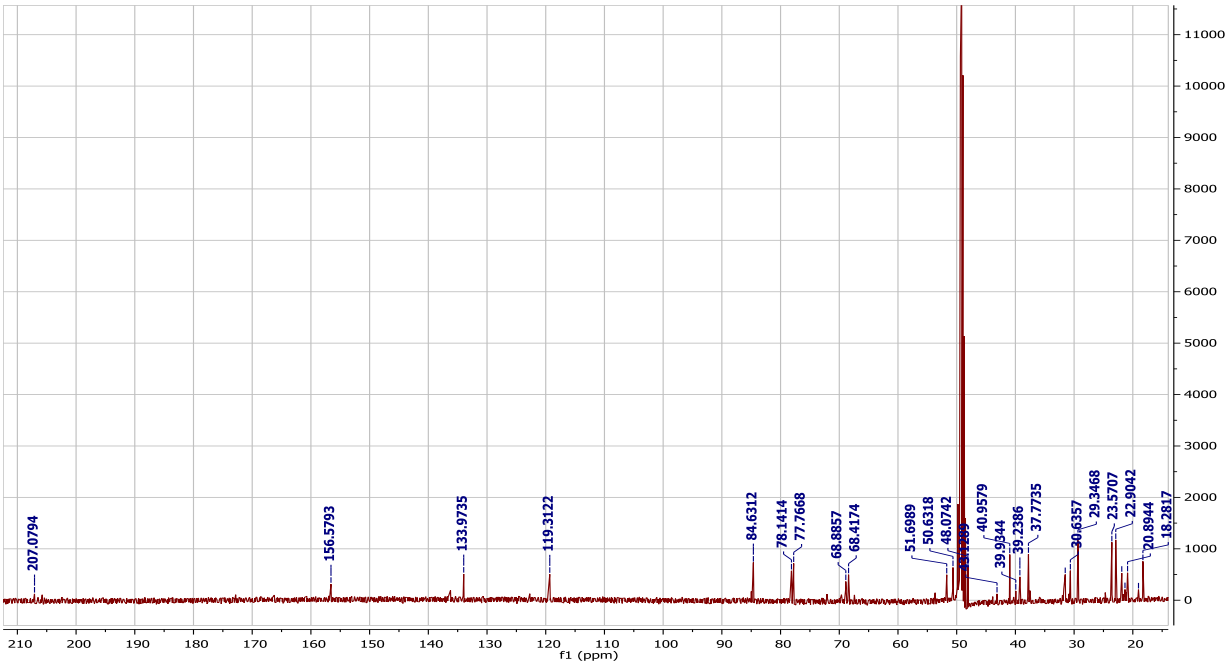


Figure 2-37 ^{13}C-NMR spectrum of compound **8** in methanol-d_4

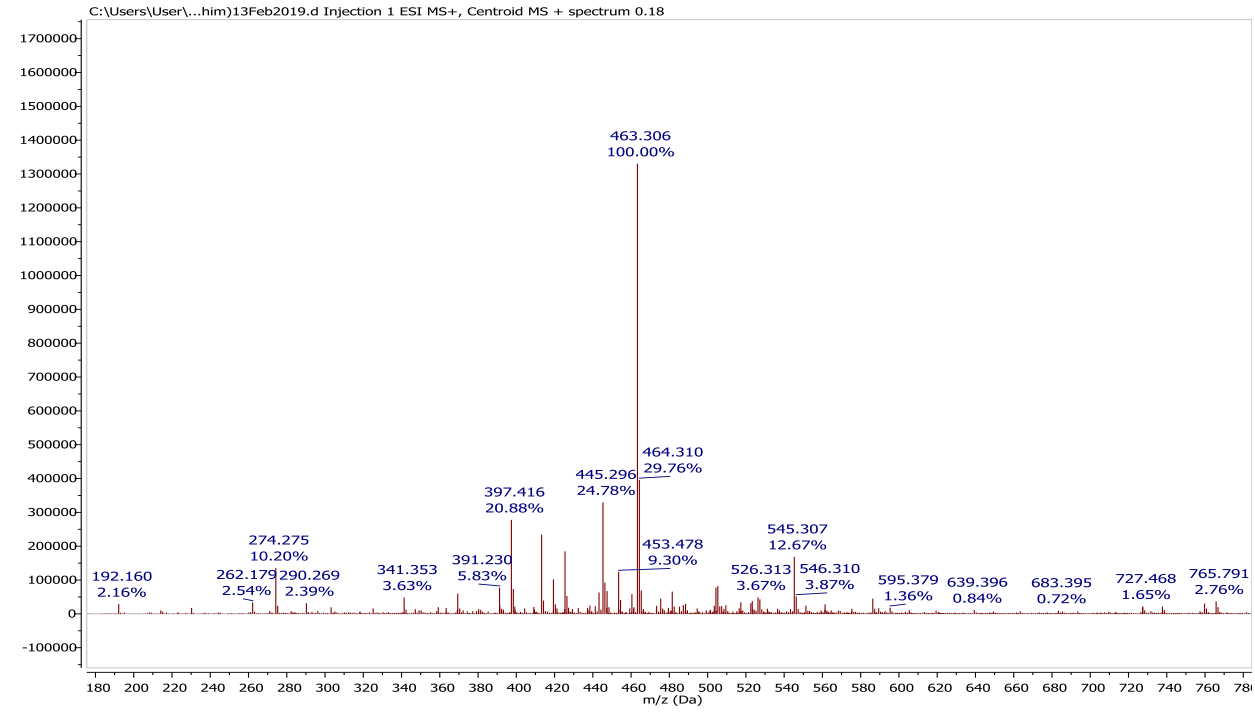


Figure 2-38 Mass spectrum of compound **8**

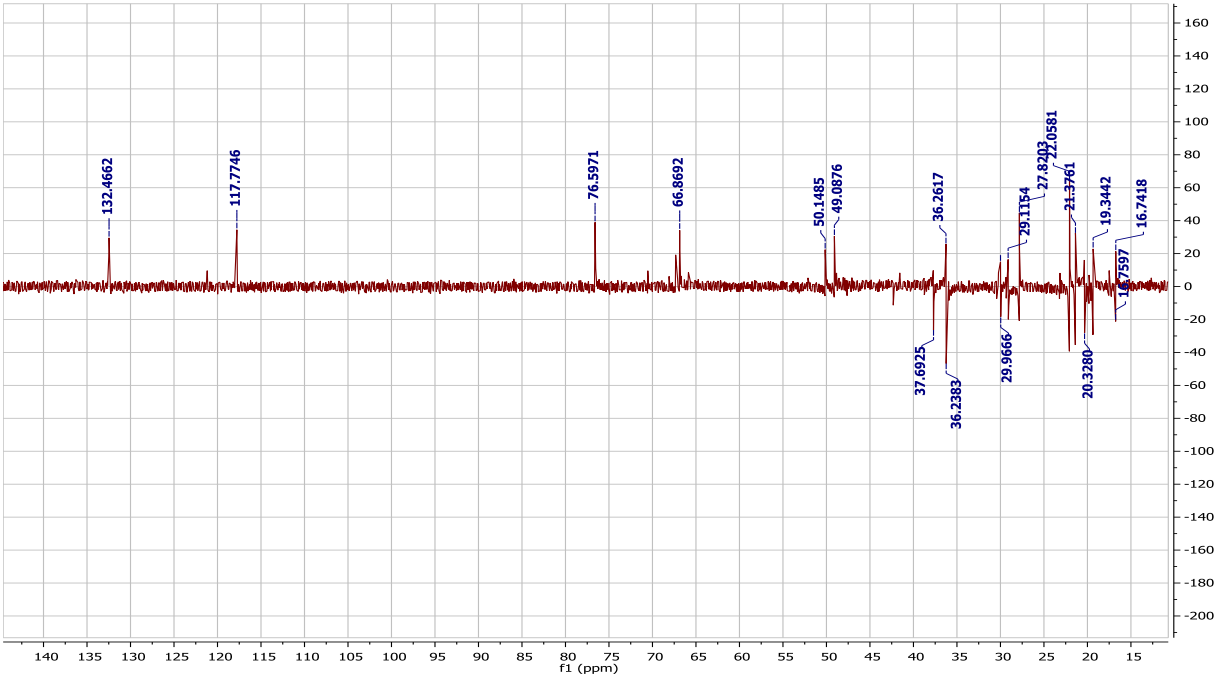


Figure 2-39 DEPT-135 spectrum of compound **8** in methanol-d_4

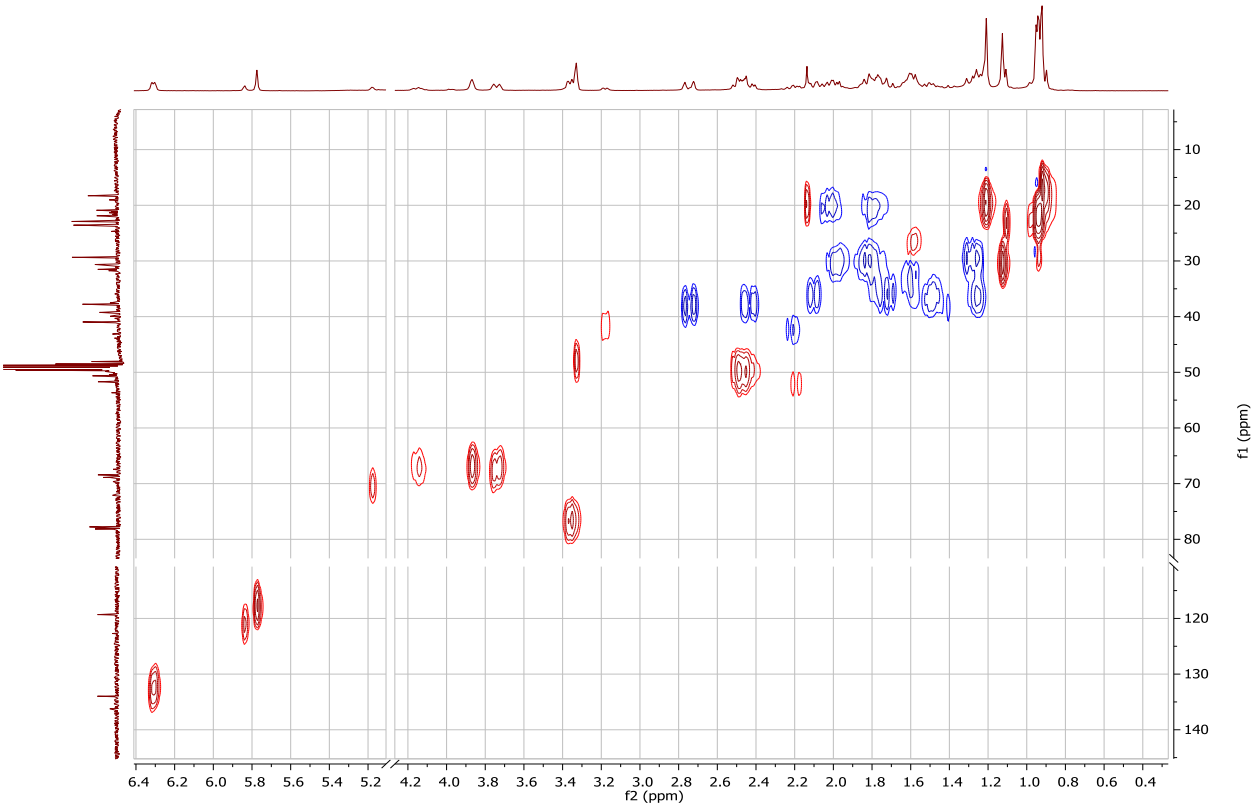


Figure 2-40 HSQC-NMR spectrum of compound **8** in methanol-d_4

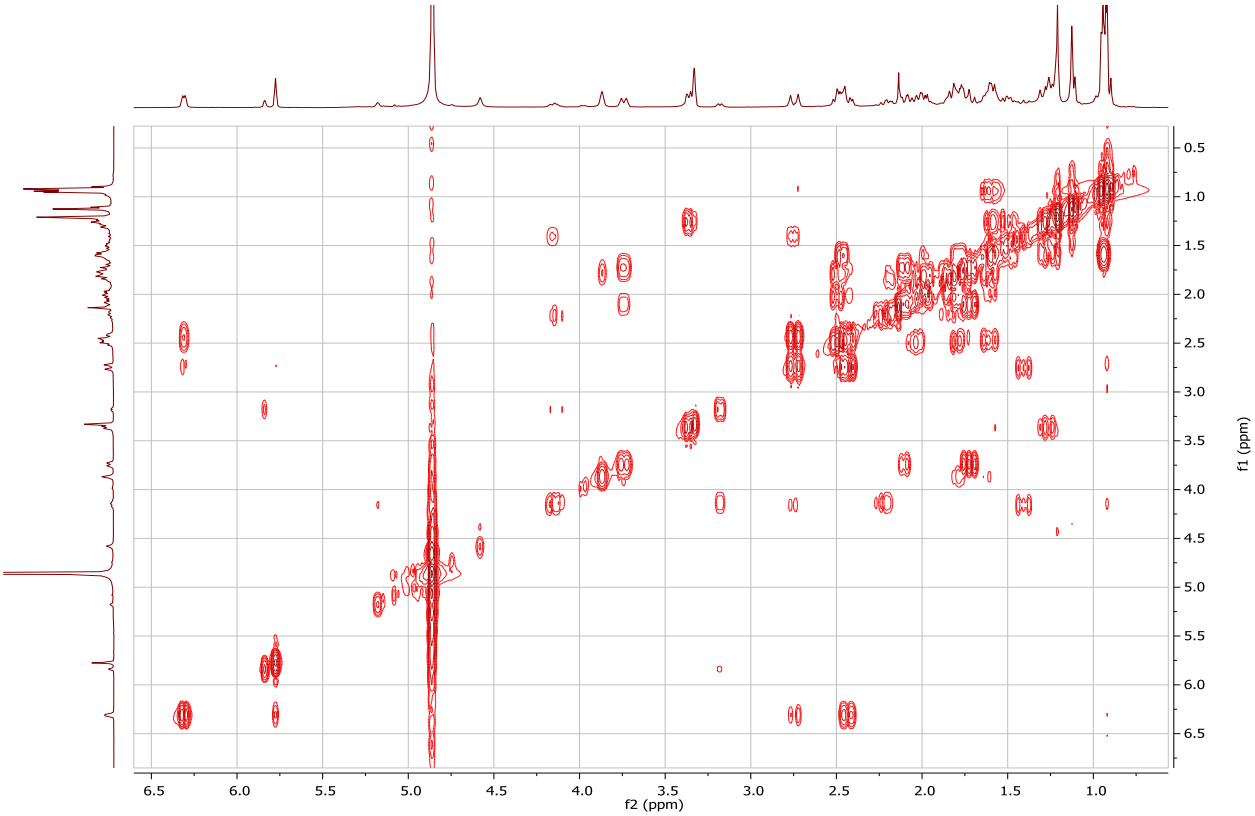


Figure 2-41 COSY-NMR spectrum of compound **8** in methanol-d_4

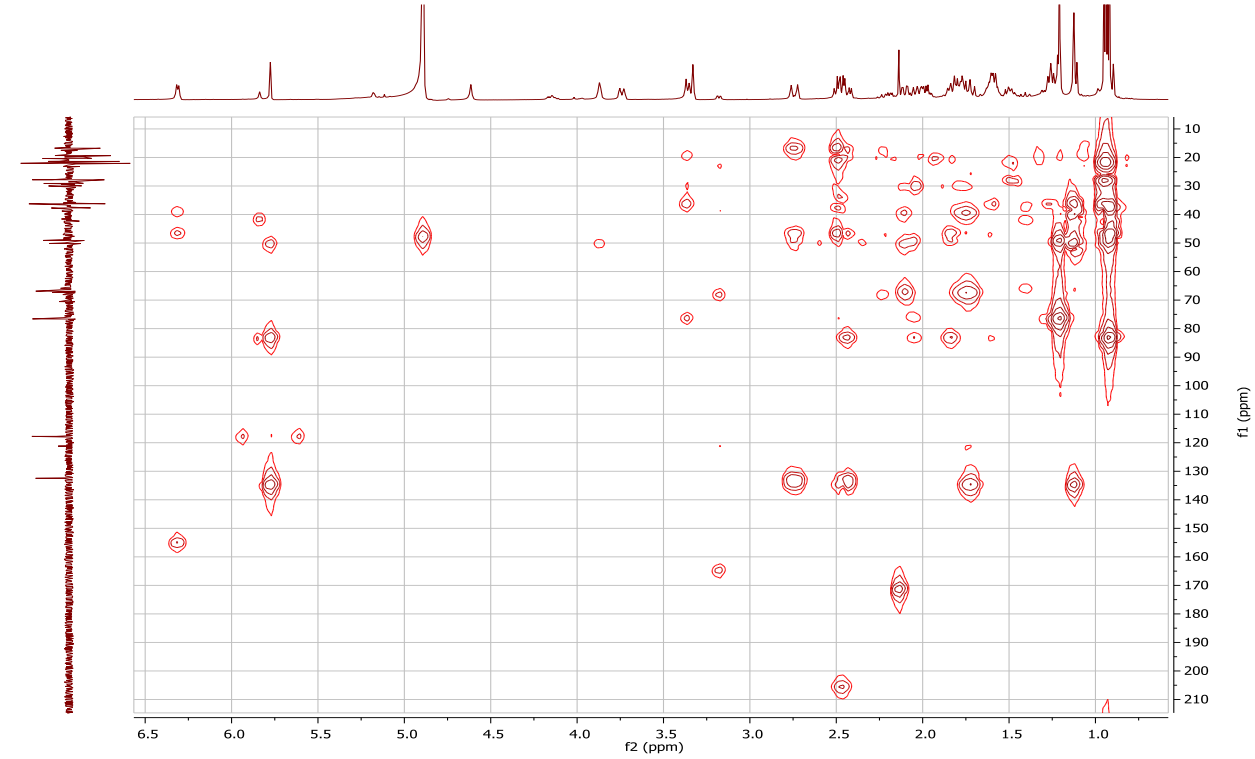


Figure 2-42 HMBC-NMR spectrum of compound **8** in methanol-d_4

2.3.2 CYP inhibition activity

C. vaga and all of its isolated compounds in this study (except compound **5)** were tested against Cytochrome P450 (3A4 isoform) – which is one of the most significant drug metabolizing enzymes. The plant extract showed strong inhibition of CYP (3A4) with an IC_{50} value of 8.5 µg/ml while 20-hydroxyecdysone and ajugasterone C, the major compounds, did not show inhibition of CYP (3A4). Compounds **2**, **3**, and **8** showed moderate inhibitions whereas **4** and **6** did not show any inhibition for CYP3A4 at all (**Figure 2-44**). No inhibition test was carried out for compound **5** because of its low yield (2.2mg). The concentration response curve of compounds **2**, **3**, **8**, and the plant extract shows the inhibition in a concentration-dependent way (**Figure 2-43**). The IC_{50} values of active samples were (75 $\pm$ 10), (41.5 $\pm$ 8.5), (85 $\pm$ 10), (8.5 $\pm$ 0.0) µg/ml, for compounds **2**, **3**, **8**, and the extract, respectively.

Overall, this finding of *C. vaga's* capability, to inhibit CYP (3A4) – one of the essential isoforms among Cytochrome P 450 enzymes – is an essential indicator for actual drug-herbal interaction. Further studies should be conducted on *C. vaga* and other significant Cytochrome P isoforms should be included. Also, these results reveal a strong inhibition activity of the plant extract with a big gap relative to the activity of its constituents. That may express the presence of other chemical compounds that were not isolated in this study, or the isolated compounds together could possesssynergic inhibition effects— which lead to an increase in the plant extract inhibitory effect.

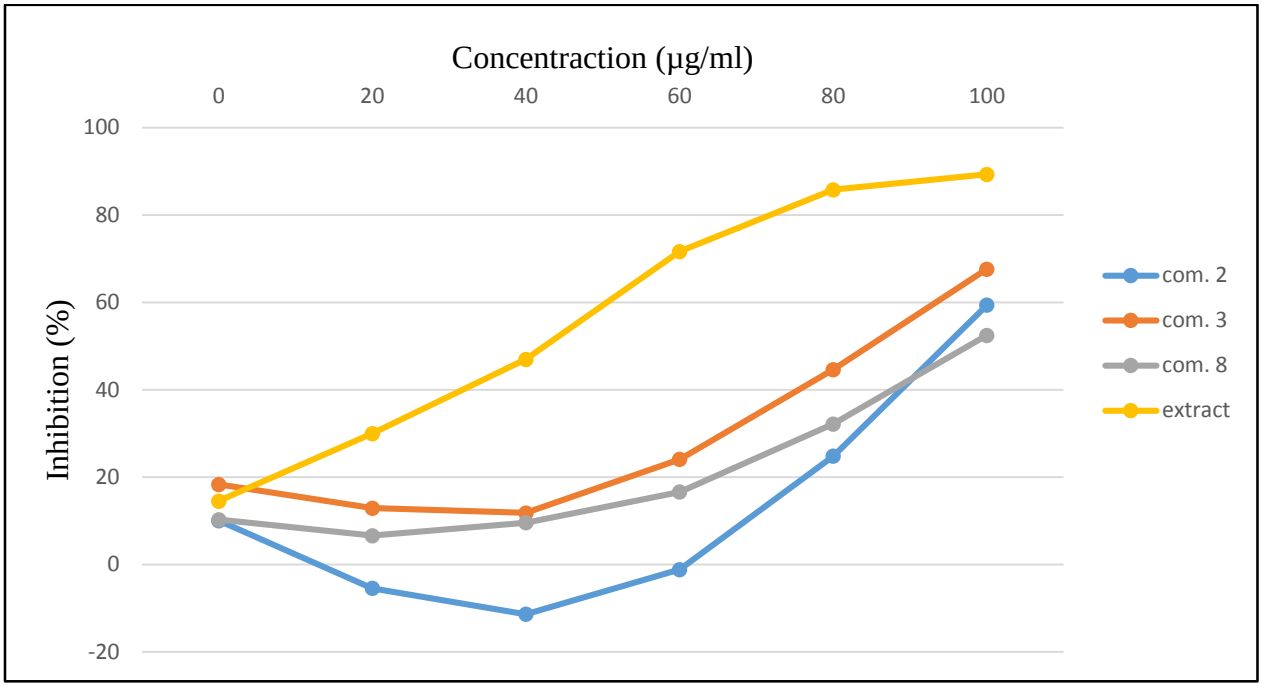


Figure 2-43 Concentration Response Curve

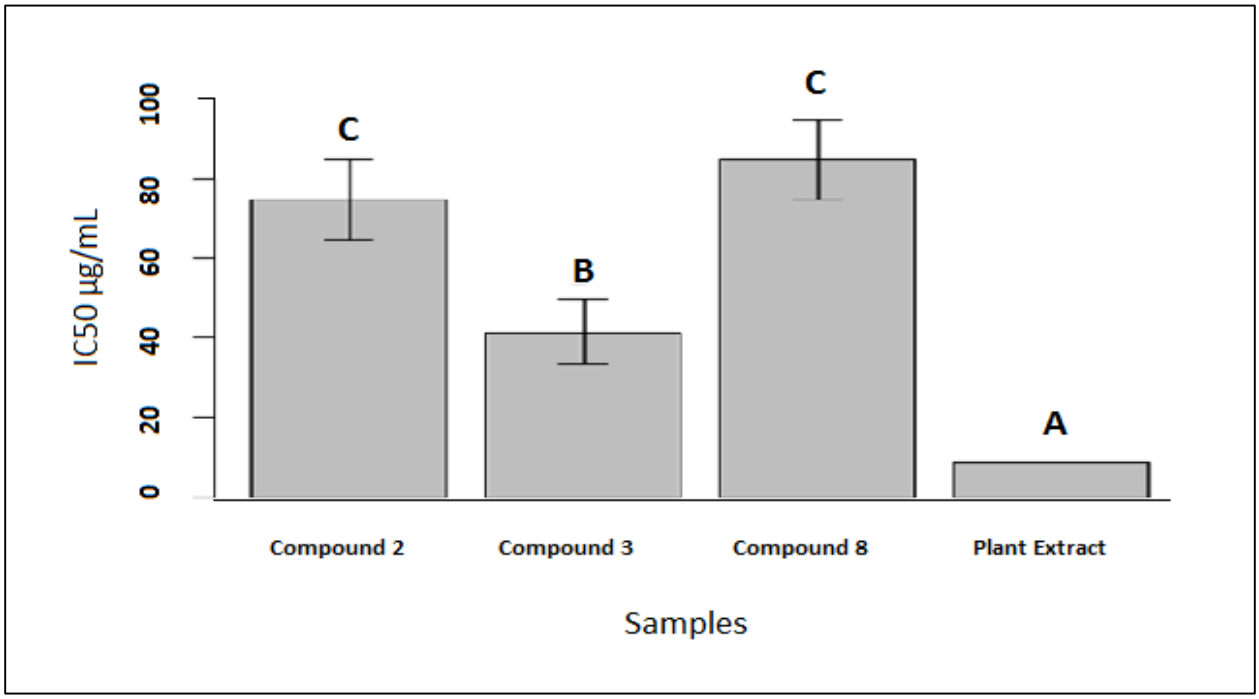


Figure 2-44 IC_{50} values of CYP (3A4) inhibition by *C. vaga* and its constituents

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